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PRIMARY MENINGEAL MELANOSIS

A Clinico Pathological Report of Two Cases

By

LEON TVETEN

Received 28 iii 64

Primary melanosis of the central nervous system is a condition in which melanin containing tumour cells arise within the meninges and spread throughout the subarachnoidal space The base of the brain and the ventral aspect of the brain stem are the main sites of involvement Obstruction of the basal cisternae is likely to occur and the effect upon the brain bears a close resemblance to that seen in any form of chronic meningitis with dilatation of the ventricular system (*Fanconi* 1956) The brain tissue is invaded by perivascular extension of the tumour growth along the perforating blood vessels

Destruction of the nervous tissue by a diffuse tumour infiltration or by circumscribed tumour nodules often results in obscure clinical pictures The disease has rarely been recognized during life and the diagnosis has been based on autopsy findings In a thorough search through the American and European literature covering the last 100 years *Pappenheim & Bhattacharji* (1963) only found 45 cases of primary melanoma in the central nervous system None of these cases had been diagnosed without surgical intervention or autopsy In addition ten more cases were described as belonging to a different category In these cases a diffuse melanosis was present in the meninges combined with numerous extensive sometimes confluent skin naevi According to a number of previous authors such a separation has been considered too rigid because a meningeal melanosis may develop independantly of the skin foci (*Bouton* 1958) This opinion is favoured by the work of *du Shane* (1943) who demonstrated the origin of the melanoblast from the neural crest

Pigmented patches in the leptomeninges may be combined with skin naevi (*Lecourturier et al* 1939) This neurocutaneous pigmentation has been recognized as a familial condition transmitted as a Mendelian dominant (*Touraine* 1949) and runs usually a benign course

The features of the *non familial* types are often more suggestive of a neoplasm The condition is very rare and only a few cases have been

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The features of the non familial types are often more suggestive of a neoplasm The condition is very rare and only a few cases have been

reported in new-born (*Grahl* 1906, *Wilcox* 1939, *Russel* 1949, *Ketels-Harken* 1963)

This report deals with two cases of primary melanosis in the central nervous system belonging to the non-familial type In one case a wide spread involvement of the leptomeninges and the nervous tissue was present combined with numerous naevi and extensive skin pigmenta tion Clinically the condition was misinterpreted because of the obscure neurological manifestations Distinct histological evidence of malignan cy was found at autopsy The second case represents the typical find ings in a newborn

CASE REPORTS

Case No 1 Female 9 Years

There was nothing remarkable in the family history, no records of inherent diseases

The patient had been in good physical and mental condition until 6 months prior to admission to the neurosurgical department Rikshospitalet in July 1951 She then gradually became mentally altered with emotional and intellectual disturbances

She was semicomatose when brought to the local medical department and ... diately transferred to Rikshopitalet with the diagnosis of a cerebral tumour

On examination the girl was found to be in an extremely bad condition, thin and dehydrated She was slow cerebrated and had poor articulation She complained of headache and difficulty in swallowing

skin of her
on her back

Neurological examination revealed a markedly sensory dysphasia a right sided hemiplegia and a sligth bulbar paresis Nothing was found on examination of the eyes

Laboratory Investigations

displayed no abnormalities
increased total protein (Heller 1/200+) and a the cells was not recorded)
ere generalized dysrhythmia with an irregular mporal region suggesting an expansive lesion

X ray of the cranium was normal

Pneumoencephalography and pneumoventriculography revealed no abnormalities but black discoloured leptomeninges were noticed through the bur holes

A left sided carotid angiography showed normal intracranial arteries

Course

The condition improved remarkably during the first few weeks of her stay until she again developed headache and nausea and started vomiting She became hyper th Death occurred in status ep



Fig 1

Case 1 Black staining tumour cells throughout the leptomeninges in various parts of the brain in the dentate nuclei and the pons

Autopsy

Autopsy (RHO 235 51) was performed 30 hours post mortem

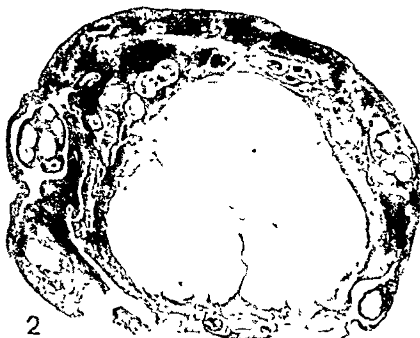
The visceral organs showed no abnormalities The eyes were unfortunately not examined

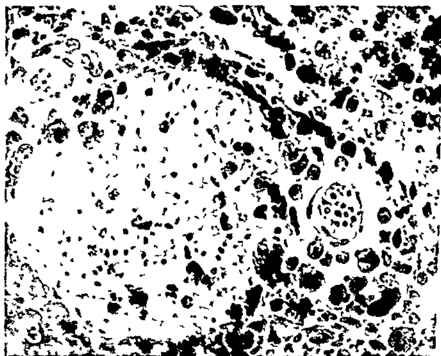
The brain weighed 1050 grams Already at gross examination of the brain a striking feature was a diffuse brownish black discoloration of the leptomeninges covering the brain and brain stem the cerebellum the spinal cord and the spinal nerve roots The brain was reexamined after 14 days fixation in 10 per cent formalin [illegible] dark [illegible] altera

Microscopic Examination

Sections from representative parts of the brain were embedded in paraffin They stained positive with ammoniacal silver nitrate and *Lillie's* Nile Blue method for melanin The stains for iron and gall pigment were negative Frozen sections stained with Sudan revealed no fat

The involved areas yielded uniform histological pictures with greatly thickened meninges infiltrated by pigmented stellate cells and non pigmented polyhedral clear cells They varied in size and shape The rounded or polyhedral cells were





Figs 2 3

Fig 2 Case 1 Cut section of the spinal cord with thickening and black staining of of the leptomeninges Ammoniacal silver nitrate stain

Fig 3 Case 1 Tumour invasion in a spinal nerve root showing cellular anaplasia Ammoniacal silver nitrate stain × 360

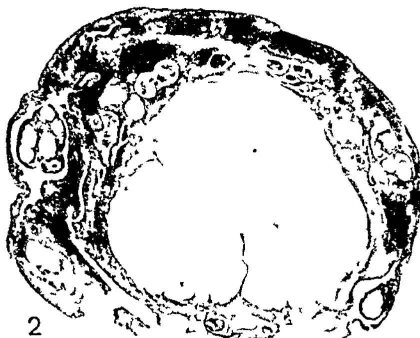


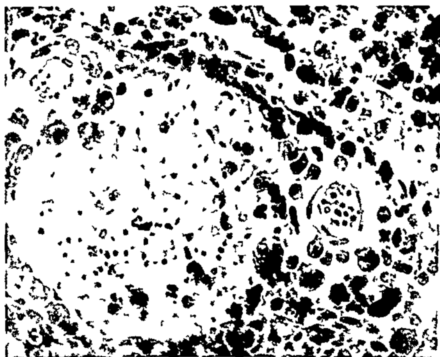


Figs 4 5

Fig 4 Case 2 Spotted dark staining of *cerebellar cortex* dentate nucleus and white matter Ammoniacal silver nitrate stain

Fig 5 Case 2 *Pigment* deposits in basal part of the pons most marked in the white matter Ammoniacal silver nitrate stain





Figs 2 3

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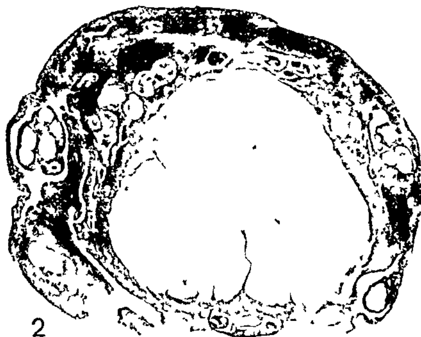


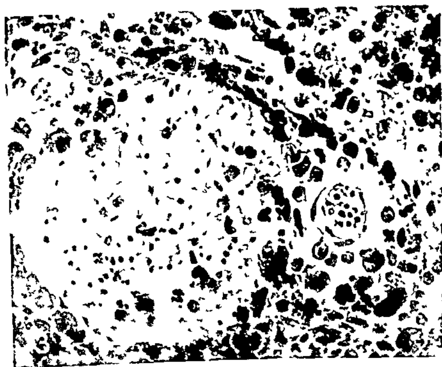


Figs 4–5

Fig 4 Case 2 Spotted dark staining of cerebellar cortex dentate nucleus and white matter Ammoniacal silver nitrate stain

Fig 5 Case 2. Pigment deposits in basal part of the pons most marked in the white matter Ammoniacal silver nitrate stain.





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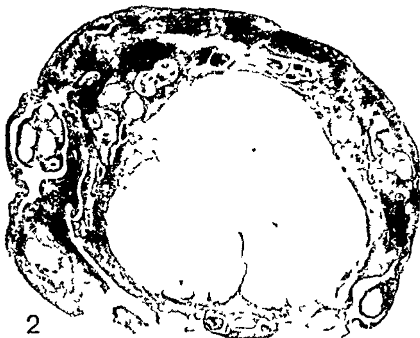


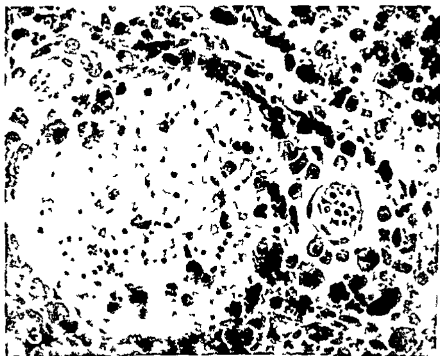


Figs 4 5

Fig 4 Case 2 Spotted dark *staining of cerebellar* cortex, dentate nucleus and white matter Ammoniacal silver nitrate stain

Fig 5 *Case* 2 Pigment deposits in basal *part of the pons* most marked in the white *matter* Ammoniacal silver nitrate stain





Figs 2 3

Fig 2 Case 1 Cut section of the spinal cord with thickening and black staining of of the leptomeninges Ammoniacal silver nitrate stain

Fig 3 Case 1 Tumour invasion in a spinal nerve root showing cellular anaplasia Ammoniacal silver nitrate stain × 360

arranged in sheaths, rows or grouped around the blood vessels. Giant cells containing multiple small nuclei in close apposition were a common finding. Other bizarre cells were also seen. In most areas the cells were stuffed with large amounts of melanotic pigments that often completely obscured the nuclei. Deposits of extracellular pigment in variable amounts were scattered at random. Cellular anaplasia and frequent mitoses were conspicuous features in some areas.

Perivascular extensions of the tumour tissue were found in many parts of the brain, the cerebellum and the brain stem. *Small necrotic foci were present* in the nervous tissue adjacent to tumour invasion. Involvement of nerve roots was also found (Fig. 3). The anterior horn cells of the spinal cord showed chromatolysis and swelling of the cell body.

Case No. 2. Male, Newborn

A boy, born to a 18 years old primipara April 63 at Rikshospitalet, was delivered

[illegible]

[illegible] within 24 hours.
A clinical diagnosis of an intracranial haemorrhage was suspected.
There was nothing remarkable in the family history.

Autopsy

[illegible]

in [illegible] were seen
ar[illegible] atelectatic

cer[illegible]
bra[illegible]

[illegible]

Microscopic Examination

Paraffin *embedded* sections and frozen sections were stained in the same [illegible]
previously described [illegible]
mented and [illegible]

[illegible]

DISCUSSION

Immature round or polyhedral, melanin forming cells, the so-called melanoblasts, may be seen microscopically in the meninges as early as the first few months of life (*Gibson et al.* 1957). They arise in the neural crest. These cells contain the enzymes tyrosinase or Dopa-oxidase which are responsible for the conversion of tyrosine or dihydroxyphenylalanine (Dopa) into melanin. Tyrosine is considered the precursor of the melanin and Dopa is regarded as the first stage of oxida-

Figs 6 7

Fig 6 Case 2 Pigmented cells in the cerebellar folia partly extending along the vessels Ammoniacal silver nitrate stain × 160

Fig 7 Case 2 Pigmentation in the walls of precapillaries in the brain stem Ammoniacal silver nitrate stain × 160

neurocutaneous syndrome as previously described by *van Bogaert* (1948) and *Fasske* (1958).

In view of the histological appearance of both cases we feel that the malignant nature of the lesions is evident.

In the first case a clinical diagnosis might have been based on the typical cutaneous pigmentation and the alterations in the cerebrospinal fluid, especially when a "curiously dark discoloration" of the meninges was noticed during the performance of ventriculography. Further, a careful study of the cellular elements in the cerebrospinal fluid might have disclosed the presence of melanin pigment. While xanthochromic spinal fluid is recorded in a high per cent of cases with meningeal melanosis, the finding of melanin pigmented cells seems to be exceptional, most probably because they are not looked for. In a recent report of primary meningeal melanoma in a 61 years old man, *Mark* (1963) has briefly discussed the criteria of the diagnosis and the findings in the cerebrospinal fluid.

No characteristic clinical signs or symptoms have been attributed to the disorder. Fainting spells, epileptic seizures, headache, nausea and mental disturbances have been recorded in a relatively great number of the cases. A progressive myelopathy of the tumour type may occur when the spinal cord is involved. A chronic meningopathy of obscure aetiology also occurs.

Signs of nervous tissue involvement may appear at any age. According to *Gibson et al.* (1957) the age incidence of meningeal melanomas has the main peak at 30–39 years and about 25 per cent of the cases die before they attain the age of 20. No causal therapy is yet available. Neurosurgical intervention usually seems to facilitate a spreading of the tumour cells.

SUMMARY

Two cases of primary meningeal melanosis of the non familial type are described. One case, a 9 years old girl, disclosed ext[illegible]

[illegible] manifestation. She died 6 months after the onset of the symptoms.

The second case represents the typical findings in a newborn. He died within 24 hours after birth due to respiratory disturbances. In both cases the pathological findings were most severe at the typical sites of predilection, i.e. the base of the brain and the brain stem.

Microscopical evidence of malignancy was present in both cases. The clinical features and the pathological findings of the disease are discussed.

tion of tyrosine by tyrosinase A positive Dopa reaction consists of *blackening of the cytoplasm of the cells which contain the enzyme* This test which is of great clinical interest, has to be performed on frozen sections of fresh tissue Unfortunately in our cases no fresh tissue was present at the time the diagnoses were established

Scattered pigmented cells are normally present in most parts of the pia, most numerous around the ventral aspect of the brain stem and the cord Here the pigmentation may be visible macroscopically Pigment carrying cells, the melanocytes or melanophages, have been recognized in the meninges of a foetus of 5½ month (*Farnell & Globus* 1931) The melanocytes are derived from the melanoblast They are characterized by their stellate shape with two or more dendritic processes and they contain numerous light or dark brown cytoplasmic granules

The melanophages are probably cells of the reticuloendothelial system which have phagocytized melanin

It is generally accepted that primary meningeal melanosis or melanomas arise from pial melanoblasts A simple hyperplasia, devoid of neoplastic feature may occur, though examples of benign hyperpigmentation of the meninges are rare in the literature *Gibson et al* (1957) in a review of 66 cases of primary melanosis of the central nervous system, including two of their own, found a wide variety of cellular form ranging from remarkably benign types to others displaying distinct histological evidence of malignancy In some cases there was no infiltration of the brain, but in others intracerebral tumour masses were distributed in different parts of the brain Prolongation of tumour into the cerebellar white matter and into the roof nuclei of the fourth ventricle were noted particularly in some of the infant cases These findings also were conspicuous features in the present cases

Most authors agree that a seeding of the tumour throughout the subarachnoid space may be found, but that this occurs as a late event In our patient, case 1, a seeding seemed to have taken place from a primary focus at the base of the brain or the brain stem

Extracranial metastases possibly also occur (*Margulies & Sabbia* 1962), *though this is not generally accepted Much of the discrepancy* in the numbers of reported primary melanoses of the central nervous system is due to exclusion of cases in which extracranial metastases or skin naevi were recorded Some authors (*McFarland & Truscott* 1961) have stressed the possibility of metastases from seemingly quiescent skin naevi

In the present cases it is our opinion that the localization and the distribution of the process within the nervous tissue strongly favour a meningeal origin of the melanosis There was no evidence of malignant cutaneous foci or involvement of other parts of the body Unfortunately the eyes were not examined but no ocular symptoms or signs had been noted *during life* The symmetrical pigmented dermatomes recognized in the first patient also tally very well with a congenital

neurocutaneous syndrome as previously described by *van Bogaert* (1948) and *Fasske* (1958)

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No characteristic clinical signs or symptoms have been attributed to the disorder Fainting spells epileptic seizures headache nausea and mental disturbances have been recorded in a relatively great number of the cases A progressive myelopathy of the tumour type may occur when the spinal cord is involved A chronic meningopathy of obscure aetiology also occurs

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Microscopical evidence of malignancy was present in both cases The clinical features and the pathological findings of the disease are discussed

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CONGENITAL NEUROCUTANEOUS SYNDROMES

A Clinico-Pathological Report of Neurofibromatosis in a Newborn and a Fully Developed Tuberose Sclerosis in a 20 Months Old Girl

By

LEON TVETEN

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Congenital neurocutaneous syndromes are conditions in which both skin and brain are involved in developmental anomalies. The best known of these disorders are neurofibromatosis or von Recklinghausen's disease, tuberose sclerosis or Bourneville's disease, neurocutaneous melanosis, retinocerebellar haemangiomatosis or Lindau's disease and encephalofacial angiomatosis or Sturge-Weber's disease.

They are considered to be based on a maldevelopment of the primordeal neuro-epithelium or of cells migrating from the neural crest to various parts of the organism. Anomalies of mesodermal nature such as angiomas and various visceral manifestations are possibly provoked by the same though usually unknown mechanism. Other authors believe that they are secondary to the ectodermal deficiency (*Benda* 1959).

A familial occurrence transmitted as an inherent dominant has been recognized in a great number of the patients with neurofibromatosis (*Neuhaus* 1948), tuberose sclerosis (*Borberg* 1951) and neurocutaneous melanosis (*Touraine* 1949). The genetical character in families with Lindau's disease is also well established (*Russel & Rubinstein* 1959), while the question of an hereditary transmission of Sturge-Weber's disease is still unsettled (*Norman* 1961).

Although the various conditions are genetically determined entities, their manifestations at times may display striking similarities. More than one syndrome may appear in the same family and even in one individual. Whether this is due to gene mutations (*Crowe et al.* 1956) or to an influence of different genes upon the same embryonic layers (*Borberg* 1951) is still a matter of argument.

Other congenital defects are commonly associated with the syndromes. Meningocele, spina bifida, syringomyelia and bony anomalies may

be encountered linking the neurocutaneous disorders to the group of dysraphic maldevelopments

The cutaneous and neurogenous lesions are represented by a number of pathological changes and clinical symptoms The skin lesions vary from small, seemingly quiescent pigmented moles to ulcerous tumour nodules The involvement of the nervous tissue may give rise to more or less extensive motor and sensory impairment Epilepsy and mental disturbances are common features

All the syndromes quoted, except Lindau's disease, may be recognized at birth or in the early childhood However, pathologically verified cases in newborn or infants are rarely reported in the literature In a recent study of neurocutaneous melanosis or primary meningeal melanosis we could only collect four cases from the literature and added one of our own (*Tveten* 1965) *Potter & Parrish*'s (1942) and *McKeown & Frazer*'s (1961) cases of neurofibromatosis are to our knowledge the only two described in newborns A *fully developed tuberose sclerosis* below the age of 4 to 5 years is an ucommon finding

This paper concerns the clinical and pathological findings in a premature, newborn female with a widespread neurofibromatosis and a 20 months old girl who suffered from a fully developed tuberose sclero sis from the age of 6 months

CASE REPORTS

Case 1 von Recklinghausen's Neurofibromatosis

Newborn premature female A 27 years old healthy primipara was admitted St Joseph's Hospital Fredrikstad in July-63 due to ablatio placenta There was nothing remarkable in the family history The pregnancy had been uneventful

About six weeks prior to the calculated term she delivered a girl weighing 1650 grams and 40 cm in length The placenta was somewhat enlarged of firm consistency and a patchy greyish white colour The child cried immediately but showed soon respiratory difficulties and became flabby A large ulcerous tumour was present on the right side of her neck and multiple small tumours were recognized in the skin of her body Death occurred 28 hours following delivery

Autopsy (RHO 253/63) was performed at the Institute of Pathological Anatomy Rikshospitalet 80 hours post mortem

A subcutaneous tumour measuring 5 cm in diameter was found in the right supra clavicular region The overlying tissue was necrotic and a central funnel shaped ulcer was present The surrounding tissue was firm indurated and adherant to the tumour

Both truncus and limbs displayed multiple small round or spindle shaped soft or firm subcutaneous tumour nodules of varying size They were scattered or in groups sometimes situated along the course of small peripheral nerves The cut surface of the tumours had a greyish-white or pink colour sometimes with central

ut sections dis

in

were observed

The brain showed a simple convolutional pattern corresponding to the premature stage The cord revealed no abnormalities

The spinal nerve roots and the main nerve truncs were found enlarged m stly marked in the left cervico thoracal (Fig 1) and the lumbar regions Multiple tum ur



Figs 1 2

Fig 1 D rsal view of the opened spinal canal sl wing a fusif rme enlargement in the upper thoracic region an l greatly thickened nerve roots m stly marke l on the left s de

Fig 2 Mult ple tumour nodules along the nerves f the lumb ar an l sacral nerve trun s



F gs 3 4

Fig 3 Subcutaneous neurof broma in the left supraclavicular region destroying the overly ng dermis Bodian stain × 80

Fig 4 Neurofibroma in the pancreas inf ltrating the glandular tissue Haematoxy l n eosin × 80





Figs 5–6

Fig 5. A nerve root ganglion showing neurofibromatous changes Bodian stain × 80
Fig 6 Rhabdomyomas in the right ventricle of the heart

nodules extended along the peripheral nerves into the deeper parts of the erector trunci and the muscles of the glutear region (Fig 2)

Microscopic examination Samples from the affected organs were fixed in 4 per cent formalin, embedded in paraffin and stained with haematoxylin-eosin, Weil's myelin stain and Bodian's silver stain The brain, the cerebellum and the brain stem were cut following 14 days fixation in 10 per cent formalin and paraffin sections were stained with gallocyanin-eosin

Sections from the tumours of the skin (Fig 3) the stomach, the pancreas (Fig 4) the peripheral nerves spinal nerve roots and spinal root ganglia (Fig 5) all displayed mainly the same histological picture with interwoven bundles of bipolar, spindle cells with ovoid or rodshaped nuclei and a pale staining cytoplasm Most of the tumours were surrounded by a thin connective tissue capsule while others were merging with the surrounding tissue, however, rather well demarcated Areas with a loose texture, sparse cells and a homogenous intercellular substanse changed with others rich in fibres and areas with closely packed cells In silver stained sections fragments of axis cylinders were sometimes found Mitoses occurred quite frequently In the central areas there was some necrosis No obvious cytological signs of malignancy were recognized

Sections from the brain and cerebellum displayed a poor differentiation of the cortical cells corresponding to the stage of prematurity No tumorous changes or glial alterations were seen

Case 2 Tuberose Sclerosis

Female, 20 months old In the family history were no records of inherent dis-
very was reported to have
47,5 cm
was noted She never suf-
quite suddenly developed
onvulsive treatment given

She was admitted to the Childrens Department, Rikshospitalet Small teleangiectatic spots were observed in the skin on her chest The clinical examination including opthalmoscopy failed to reveal signs of any other organic lesion X ray of the cranium was normal

Electroencephalography indicated a diffuse cerebral lesion with epileptogenic potentialities

The cerebrospinal fluid was normal

During her stay she had repeated small and larger convulsive attacks, and for further observation she was transferred to Statens sykehus for epileptikere, Sandvika, Bærum

During the following months a marked developmental retardation was noted The seizures were only poorly controlled though different anti convulsive compounds

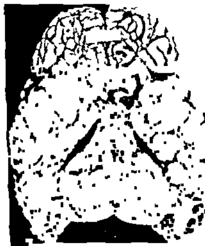


The lungs weighed 200 grams The lobes were partly atelectatic There was no exudate in the pleura or the pericardium

The heart weighed 61 grams Several firm from pea to nut meg sized, greyish red tumour nodules were found in the myocardium and some of these projected into the right ventricle (Fig 6)

A bean sized tumour was localized to the septum of the left ventricle Cut section revealed a homogenous, quite firm tumour sharply demarcated from the adjacent, normal looking myocardium

The kidneys weighed 60 grams Several solitary, peasized cysts appeared on the surface and in the deeper parts of both kidneys The cysts contained a colorless and limpid fluid





Figs 7–8

Fig 7 Base of the brain showing widely spread tuberose changes in the cortical gyri

Fig 8 A low power view of one of the tumour nodules in the mycardium Heidenhain s Iron haematoxylin eosin stain × 80





Figs 5–6

Fig 5 A nerve root ganglion showing neurofibromatous changes Bodian stain × 80

Fig 6 Rhabdomyomas in the right ventricle of the heart.

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Case 2 Tuberose Sclerosis

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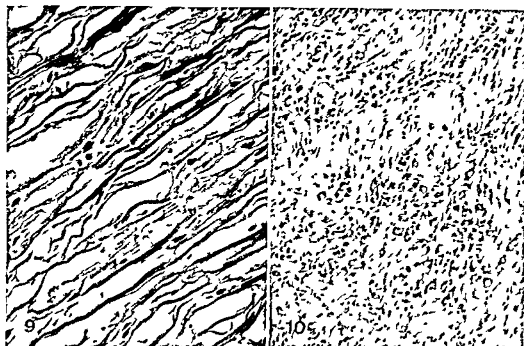
Figs 11 12

Fig 11 Section from right parietal region with cortical and subcortical tubera Weil's myelin stain

Fig 12 Frontal section of the brain showing the cortical tubera with corresponding demyelination in the white matter Weil's myelin stain

The histological picture of the brain tumours revealed an enormous overgrowth of glial cells mostly fibrillary astrocytes of different size and shape Giant cells were mostly localized to the outer zones of the tubers The normal cortical lamination was disrupted or entirely missing The remaining neurones most often displayed moderate pyknotic changes

Groups of large irregular cells with a swollen homogenous eosinophilic cytoplasm and one to three round or oval eccentric nuclei were conspicuous findings mainly in the subependymal nodules (Fig 10) The Holzer stain revealed a dense



Figs 9 10

Fig 9 High power view from the same nodule Note the cross striation of the muscle fibers Heidenhain s Iron haematoxylin eosin stain × 400

Fig 10 A subependymal nodule revealing dense fibrillary gliosis and a group of large irregular cells Gallocyanin eosin × 360

The other internal organs showed no abnormalities

The brain weighed 1260 grams The leptomeninges were thin and translucent The convolutional pattern was altered by multiple yellowish white areas of abnormally broad gyri slightly elevated above the adjacent normal looking gyri (Fig 7) Their surfaces were flattened and smooth the consistency firm or hard The alterations were present all over the brain

The brain was cut following 10 days fixation in 10 per cent formalin Frontal section revealed multiple small and large yellowish white tumorous nodules in the cortex and the adjacent white matter Small tumour nodules were found along the walls of the ventricle mostly at the left sulcus terminalis and in the corpus nuclei caudati on the right side

No gross abnormalities were found in the cerebellum the brain stem or the spinal cord

Microscopic examination Sections from representative parts were stained with haematoxylin and eosin van Gieson elastin periodic acid Schiff (PAS) Sudan fat stain Mallory s phosphotungstic acid haematoxylin gallo cosin Holzer s stain for glial fibrils luxol fast blue Weil s myelin stain and Bodian s method for axons and neurofibrils

Sections from the skin patches showed subepidermal haemangiomas composed of multiple small thick walled vessels surrounded by sparse connective tissue A hyperplasia of the sebaceous glands was observed The epidermis was somewhat thickened

The small cysts of the kidneys were lined by low cubical epithelium The remaining kidney tissue was normal

The heart lesions were typical of the rhabdomyoma sharply demarcated from the normal myocardium by varying amounts of connective tissue (Fig 8) Large irregular tumour cells with uniform nuclei prominent nucleoli and an abundant
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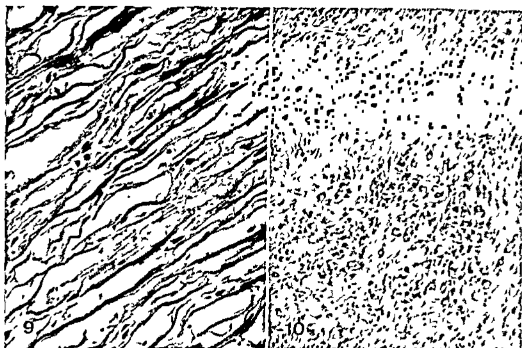
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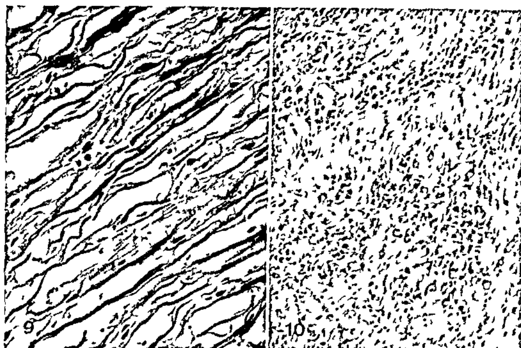
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A few, small concrements staining dark blue with haematoxylin-eosin were present in some of the nodulus of the basal ganglia

DISCUSSION

Neurofibromatosis is clinically recognized by multiple, often pedunculating tumours as well as melanin pigmented patches in the skin The original description by *von Recklinghausen* in 1882 concerned only the skin manifestations Further observations have shown that peripheral nerves in any organ may be involved In the central nervous system rootlet tumours from the spinal cord and the brain stem are fairly frequent The acoustic nerves are most often involved uni- or bilaterally Meningeomas and central glial tumours may also be associated with the disease, and when present they are usually multiple It has been stressed that when the peripheral manifestations are numerous, few or no central lesions are found (*Russel & Rubinstein* 1959)

The findings in the present case are compatible with this observation The prominent features were almost entirely confined to the main nerve truncs and the peripheral nerves A wide dissemination of tumour masses, rounded nodules and fusiform enlargements, occurred along the course of the nerves in many areas Isolated neurofibromata were present within the dermis and the subcutaneous tissue of the trunc and limbs These lesions are supposed to arise from the peripheral nerve endings (*Chao* 1959a) A large ulceration of the overlying dermis was found in the supraclavicular region, probably due to trophic disturbances, with necrosis of the skin by the pressure of the tumour

No definite malignant changes were observed

Pigmented patches of the cafe au lait type have in some cases been recognized at birth, although they usually develop later in life No such pigment anomalies were seen in the present case It should be remembered that not all skin naevi, pigmented moles or neurinomas undisputedly are manifestations of the neurofibromatosis syndrome, but they may represent abortive forms

Visceral neurogenous tumours have been described in many internal organs, both as isolated phenomena and associated with the disease (*Schmitt et al* 1961) Recently *Perea & Gregory* (1962) in reviewing the english medical literature of gastric neurofibromas found 29 cases, 5 of which were accompanied with neurofibromatosis Small-sized lesions in different internal organs may be clinically silent, and they may also remain unobserved at autopsy unless they are carefully looked for A few reports have appeared in which endocrine organs have been involved (*Benda* 1952, *Schmitt et al* 1961), most often the adrenal cortex Depending upon the site of involvement a multiplicity of endocrinological symptoms may develop, such as obesity, gigantism, precocious puberty, mentrual anomalies and others

Pancreatic lesions, as found in the present case, are rarely reported. Neurofibromatosis has been recorded associated with other types of neoplasms also deriving from the neural crest. The combination with phaeochromocytomas and ganglioneuromas has been demonstrated in a relatively high percentage of the cases (*Russel & Rubinstein* 1959). The case of *Potter & Parrish* (1942) exemplifies the close interrelationship of ganglioneuroma, neuroblastoma and neurofibroma.

The nature of neurofibromas has been a matter or argument for many years. Most authors now agree that the tumours are based upon developmental disturbances at the meso-ectodermal junction in the early embryonic stage, and that both Schwann cells and perineural fibroblasts are involved.

Although neurofibromatosis in itself is a benign disorder, the prognosis may be difficult to predict. According to *Russel & Rubinstein* (1959) sarcomatous changes are not unusual. The incidence of glial proliferation in brain and spinal cord, the frequent co-existence with other neoplasms and congenital defects, also call for the greatest care in the evaluation of the prognosis.

Bourneville (1880) gave the first description of tuberose sclerosis in a 14 months old infant with epilepsy and skin lesions on the face. His name later became connected with the syndrome. The typical clinical triad with mental retardation, epilepsy and adenoma sebaceum, however, was first recorded by *Vogt* in 1908, and it commonly has been considered a requisite for the diagnosis. Later reports have shown that one or more of the three classical symptoms are by no means always present (*Presthus* 1953, *Schnitzer* 1963).

The facial manifestations will most often give the key to the diagnosis in epileptic patients. These lesions are of naevoid character made up by hyperplastic sebaceous glands and a varying com[illegible] fibro-
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The mental deficiency varies greatly. In infants the degree of this defect often is difficult to evaluate, but the progressive course may be of diagnostic value. X ray of the skull may reveal [illegible] in the
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[illegible] The type of seizure is by no way pathognomonic but the attacks have a tendency to occur in series with free intervals of up to several years (*Presthus* 1953). The electroencephalograms show all types of abnormalities.

In the absence of the typical triad of symptoms, the diagnosis is easily overlooked, and in infants the autopsy findings are not seldom un-

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The facial manifestations will most often give the key to the diagnosis in epileptic patients. These lesions are of naevoid character made up by hyperplastic sebaceous glands and a varying component of fibroangiomatous origin. As a rule they do not appear until the age of 4 to 5 years, and often not until puberty (*Norman* 1961). Other skin lesions may also be associated with the disease. Shagreen patches and plaques, haemangiomas, vitiligo, naevi of the cafe au lait type and other pigmented spots with an extrafacial distribution have been reported (*Chao* 1959b).

The mental deficiency varies greatly. In infants the degree of this defect often is difficult to evaluate, but the progressive course may be of diagnostic value. X-ray of the skull may reveal calcifications in the ventricular walls or located to the cortical areas.

The seizures usually start within the first 2 years of life, some times they are present at birth. The type of seizure is by no way pathognomonic but the attacks have a tendency to occur in ser[illegible]
vals of [illegible]
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over[illegible] [illegible]utopsy findings are not seldom un

expected, as in the present case The skin manifestations might have been suggestive although they were not of the adenomatous type The family history was negative, and X-ray of the skull failed to show any calcification

The brain lesions are commonly recognized already on gross inspection by the presence of the pathognomonic, sclerotic patches in the gyri, the so-called tubera *Von Recklinghausen* already in 1862 published a description of these typical brain alterations His report also included multiple rhabdomyomas in the heart

Another frequent pathological finding is multiple, small tumour nodules projecting into the ventricles from the basal ganglia They are commonly found beneath the ependyma of the lateral ventricle, and the site of predilection is the sulcus terminalis When sufficiently calcified they show up in X-ray photographs, and they may be of great diagnostic value

In the present case the wide dissemination of cortical and subcortical white matter lesions as well as small subependymal tumour nodules in the basal ganglia were conspicuous

In the fully developed disease as well as in its abortive forms, tumour-like changes may be found in many internal organs, such as the heart, lungs, liver, spleen and kidneys Recently *Schnitzer* (1963) reported an adult case of tuberose sclerosis with massive renal involvement The patient had never been mentally retarded There had been no epilepsy, and adenoma sebaceum was missing At autopsy typical subependymal nodules only were present in the brain

Outside the brain, the kidneys probably are most commonly involved, according to *Critchley & Earl* (1932) in about 80 per cent of the cases The lesions usually are multiple and bilateral Two different types may be recognized a solid tumour composed of smooth muscle cells fat and blood vessels, described as angio-myo-lipomas, or a cystic tumour of fibroangiomatous type Malignant changes in the former have been reported by *Harbitz* (1912)

The presence of multiple, small cysts in the kidneys of our patient may represent an incidental finding, although they probably were of congenital nature There had been no evidence of renal impairment

The case of *Engstrom et al* (1962) showed a polycystic renal disease, a finding which the authors believed was unrelated to tuberose sclerosis

Rhabdomyomas in the heart occur in around 50 per cent of the cases of tuberose sclerosis (*Schnitzer* 1963) They are most often located to the apex of the left ventricle In the present case they were mainly embedded in the myocardium of the right ventricle Microscopically the tumours showed the morphologic characteristics recently described in detail by *Wolf & Foley* (1963) We also found the tumours rich in fat and glycogen, but unfortunately in the absence of fresh material, we have not been able to confirm the presence of oxidative enzyme activity, as obtained by these authors

The prognosis in tuberose sclerosis is to some extent depending upon the distribution of the lesions The fully d veloped condition most often ends fatally already in childhood Death is usually provoked by an inter current infection or occurs during status epilepticus

A malignant change in the brain tumours is not infrequent Intra ventricular tumours arising from the subependymal nodules may grow large distend and fill the ventricular system and the cortical nodules may undergo a malignant change and infiltrate as gliomas

SUMMARY

A brief review of the neurocutaneous syndromes is presented Clinico-pathological report of two cases is given neurofibromatosis or von Recklinghausen s disease and tuberose sclerosis or Bourneville s disease respectively The former was present in a premature newborn who died 28 hours following delivery The latter was a 20 months old girl who suffered from protracted epileptic seizures and a progressive mental retardation from the age of 6 months but the typical skin lesions were absent Clinically the diagnosis were unsettled and the autopsy findings were unexpected

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Acta path et micr 1 i 1 scan linas 63 23 27, 1965

Département de Z ologie Physiol gique Helsinki Finlande

CULTURE D'EBAUCHES DE POUMON ET D OESOPHAGE EN MILIEU SYNTHETIQUE

Par

Luis Soriano[1] et Lauri Saxén[2]

Reçu 19 v 64

Il est nettement prouve que les organes embryonnaires "*in vitro*", peuvent subir un changement morphologique s ils sont cultives en milieu synthetique (*Wolff et coll* 1953, *Stenger Haffen* 1957) De plus *Lasnitzki* (1962) a trouve que la glande prostatique de la Souris organe typiquement secreteur en culture sur milieu naturel, peut subir une metaplasie squameuse si elle est cultivee sur milieu synthetique En partant de ce fait, nous nous sommes demandé s il serait possible d observer des changements semblables dans l ébauche de poumon cultivee en milieu synthetique Dans une etude anterieure (*Soriano et coll* 1964) nous avons trouve que cet organe survit longtemps en milieu naturel et qu il se differencie alors en organe capable d élaborer des sécretions

MATERIEL ET METHODE

Des fragments d ébauches de poumon et d oesophage s nt prelevés sur des embry ns de Souris de 12 jours Ils sont constitués par l épithelium pulmonaire et l epithelium oesaphagique séparés par un tissu mésenchymateuse commun Ils sont cultives tels quels selon une methode de *Trowell* modifiée decrite ailleurs (*Saxén et coll* 1962 ...

s pre

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RESULTATS

Ce milieu sans proteines ne permet pas aux ébauches d atteindre un degre de croissance normal, toutefois la differenciation de l'epithelium est semblable a celle obtenue quand les ebauches de poumon et d oesophage sont cultivees sur milieu naturel En fait, après 5 jours de culture,

[1] Ce travail a ete fait grace à une bourse de ... Consejo Nacional de ... entine Adress actuel ... 49 bis Avenue de la ...

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Figs 3-4

Fig 3 Fbauches de poumon et d oesophage apres 15 jours de culture en milieu naturel En haut épithelium pulmonaire En bas épithelium oesophagique

Fig 4 Ebauches de poumon et d oesophage après 15 jours de culture en milieu synthetique A droite epithelium pulmonaire A gauche epithelium oesophagique



2

Figs 1-2

Fig 1 Ebauches de poumon et d oesophage apres 9 jours de culture en milieu naturel En haut epithelium pulmonaire En bas epithelium oesophagique

Fig 2 Ebauches de poumon et d oesophage apres 9 jours de culture en milieu synthétique A droite epithelium pulmonaire A gauche epithelium oesophagique

prolonged experiments the mesenchymal tissue is affected first, and only subsequently the overlying epithelium The role of the mesoderm, which will be analyzed further in subsequent recombination experiments is discussed

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11 [illegible]

les epithelia oesophagique et pulmonaire atteignent le même degre de differenciation sur le milieu synthetique et sur le milieu normal

Il faut toutefois mentionner que les explants cultives en milieu synthetique montrent une diminution progressive de la quantite de mesenchyme Tandis que dans les deux ebauches l'epithelium garde sa structure, le mesenchyme tend a disparaître au fur et a mesure qu'il vieillit (Fig 1, 2, 3 et 4)

DISCUSSION

Etant donnes ces resultats, il ne semble pas que certains facteurs nutritifs jouent un rôle fondamental dans la differenciation "*in vitro*" des rudiments pulmonaire et oesophagique En effet, cette etude nous permet de constater que la differenciation s'effectue aussi bien sur un milieu synthetique que sur un milieu normal, et ceci en depit de l'absence des proteines, vitamines et hormones dans le milieu synthetique Ce point merite quelques commentaires, puisque *Lasnitzki* (1962) cultivant la prostate dans un milieu synthetique complexe sans proteines observe une metaplasie notable apres 10 jours *Fell* (1960) a montre que ce processus pouvait ne pas exister si on ajoutait au milieu de la testosterone ou de la vitamine A

D'autre part *Stenger-Haffen* (1957) a montre que certains aminoacides jouent un rôle important dans la differenciation de la gonade, les amino-acides sulfhydriles peuvent assurer la differenciation de la gonade femelle cultivee en milieu synthetique Ce resultat pourrait expliquer le nôtre, puisque le milieu de Eagle contient quelques aminoacides sulfhydriles

De plus differents auteurs ont montre que des modifications de la composition du milieu nutritif, aussi bien que l'adjonction de differentes vitamines et hormones ou des variations de l'atmosphere gazeuse entourant les explants, peuvent changer le mode de differenciation normal des organes embryonnaires en culture (*Wolff* et *Wolff* 1952, *Wolff* et *Haffen* 1952, *Fell* et *Mellanby* 1953, *Wolff et coll* 1953, *Stenger-Haffen* 1957, *Moscona* 1959, *Lasnitzki* 1961)

Le comportement des ebauches oesophagique et pulmonaire cultivees "*in vitro*", semble dependre des potentialites intrinseques de l'epithelium et de ses rapports avec le mesenchyme correspondant En relation avec cette idée, des experiences sont en cours pour elucider le rôle du mesenchyme dans la differenciation des ces tissus epitheliaux

SUMMARY

Esophageal and lung rudiments of mouse embryos have been cultured in a chemically defined medium and their differentiation compared with that in "normal" medium *in vitro* The medium seems to allow rather good epithelial differentiation and squamous metaplasia of the oesophageal epithelium occurs as in the normal culture conditions In

prolonged experiments the mesenchymal tissue is affected first, and only subsequently the overlying epithelium The role of the mesoderm, which will be analyzed further in subsequent recombination experiments, is discussed

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SOME EFFECTS OF CATIONIC POLYMERS ON CELL ATTACHMENT AND GROWTH BEHAVIOUR

Studies with Polybrene®

By

STIG NORDLING, ERKKI SAXÉN and KARI PENTTINEN

Received 4.xi.64

In a recent work (*Nordling et al.* 1964 c) it was reported that heparin and other anionic polymers in serum containing medium greatly inhibit the cell attachment to a glass surface, and cause HeLa cells in stationary cultures to grow in dense clumps, only loosely attached to the glass surface, and not in the ordinary way in a mosaic structure in monolayers with firm attachment to the glass. Previously the observation had been made that the same effect was exerted by some fresh, individual sera (*Saxén & Penttinen* 1961 and *Nordling et al.* 1963). In contrast, cationic polymers have been reported to cause red blood cells to adhere strongly to a glass surface (*Kachalsky et al.* 1959). Others papers have established that anionic polymers prevent blood coagulation (*Shafrir et al.* 1954 and *Vaheri et al.* 1964). Polybrene inhibits the anticoagulant effect of heparin (*Preston* 1952) and other anionic polymers (*Vaheri et al.* 1964). Other cationic polymers also inhibit the anticoagulant effect of these substances (*Shafrir et al.* 1954). These facts led to the idea that cationic polymers also would neutralize the attachment inhibiting effect of both anionic polymers and fresh clumping serum and/or make HeLa cells more adhesive to glass. The present investigation was made with a view to testing these hypotheses.

MATERIALS AND METHODS

HeLa cells grown in a medium containing 30 per cent aged human serum pool in Hanks' solution were trypsinized for 30 minutes at 37° C using a final concentration of 0.16 per cent of trypsin (Difco 1: 250). All the glassware was washed in an alkaline washing solution.

The attachment experiments were made in attachment chambers (*Taylor* 1961, *Nordling et al.* 1963), which were filled with inocula of about 60 000 cells/ml in 30 per cent serum in Hanks' solution and tris buffer pH 7.2 (Sigma 7–9). If not other

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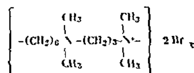
The present investigation has been aided by grants from the *Sigrid Jusélius foundation* and *President J. K. Paasikivi foundation*.

The skilful technical assistance of Mrs. *Ansa Kanerva* is greatly acknowledged.

wise stated all components of the medium and cells were pipetted together into the chambers The chambers were completely filled and incubated for stated times selected between 5 minutes and 2 hours in an incubator at 37° C, after which they were inverted The unattached cells would then drop by gravitation from the upper surface and settle on the lower The attached cells on standard areas on the upper surface and settled cells on corresponding areas on the lower surface were counted and the percentage of attached cells was calculated

In the *growth behaviour experiments* inocula of 30 000 cells in 1 ml of 30 per cent aged serum pool in Hank s solution and tris buffer were put into culture tubes (16 × 120 mm) of Pyrex glass The growth behaviour was recorded after 24 hours of

quaternary ammonium compound which can be regarded as a cationic polymer with with the following probable structural formula



It was manufactured by Abbott in an isotonic aqueous solution which contains 10 mg/ml Immediately before use this solution was diluted with Hanks or buffered saline to the desired concentration and added to the culture medium or was pipetted *on the glas surface* or used in the agglutination tests The reported concentrations are the final ones

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cium and magnesium ... same The HeLa cell concentration was about 2.5×10^{6} cells/ml The *agglutination* was recorded after 10 minutes at room temperature and compared with *the controls* without Polybrene

RESULTS

Attachment Experiments

Polybrene enhanced cell attachment in a serum containing medium, so that within 1 *hour all of the cells* were attached, as compared with 50 per cent in the *control medium, aged serum pool* without Polybrene (Fig 1) In this experiment the cells and Polybrene were put into the chambers at the same *time* If Polybrene were put into the chambers some time before the cells were added, the difference would be even more pronounced, and the cells attached to the Pol ...

... and *Nordling et al* ... it was of no importance whether or not Polybrene was present in

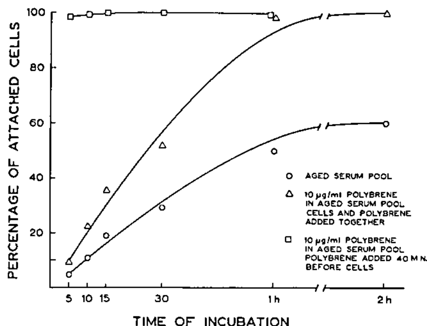


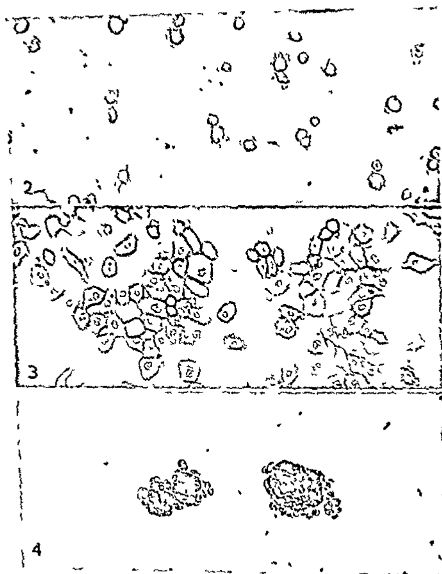
Fig 1

The effect of Polybrene on cell attachment on a glass surface The medium consisted of 10 µg/ml of Polybrene and 30 per cent aged serum pool The attachment chambers were incubated at 37° C

the culture medium, once it had acted on the glass surface In our experiments the Polybrene was allowed to act during 40 minutes The same effect was observed in all sera studied, also in fresh clumping and fresh non-clumping sera In these sera almost no cells attached on a normally heated glass surface after 10 minutes

Growth Behaviour Experiments

In low concentrations starting from 2 µg/ml to 8 µg/ml of Polybrene, the clumping effect neither of anionic polymers nor of fresh serum can be observed Polybrene also effects the morphology of the cellular growth The cells do not grow as monolayeers, but far from each other They are large and round, and the impression is gained that the mitotic activity is low ' Polybrene-effect ' (Fig 2) For comparison the growth behaviour in non clumping and clumping serum is showed in Figs 3 and 4 In somewhat higher concentrations it is toxic to cells, and in serum-free medium it appears to be toxic even in concentrations of about 10 µg/ml The "Polybrene-effect ' is exercised at the same concentrations in fresh non clumping serum and aged serum pool, but in media containing high concentrations of anionic polymers it seems that higher Polybrene concentrations are required for the ' Polybrene-effect" to occur A possible explanation is that some of the Polybrene acts direct with the polymer



Figs 2-4

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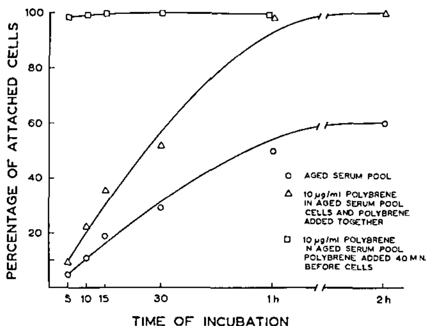


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TABLE 3

Agglutination of Red Blood Cells by Polybrene in a Serum Free Medium

Polybrene concentration µg/ml	Agglutination of red blood cells
0	—
2	—
3	—
4	(+)
5	+
6	+
7	+
8	++
10	++
25	+++

— = no agglutination
(+) to +++ = degree of agglutination

DISCUSSION

As far as we know earlier reports on the effects of cationic polymers on cells *in vitro* have been concerned only with poly α-amino acids and their effects on red and white blood cells, with the exception of a short note on the effect of salmine and poly-L-lysine on the attachment of *Hyfendis* A I cells in a paper by *Lieberman & Ove* (1958). *Lisnell & Mellgren* (1963) have studied the effect of Protamine sulphate on monolayers already established. The biological effects of poly α-amino acids have recently been reviewed by *Sela & Katchalsky* (1959) and *Katchalsky* (1964).

The present results show that Polybrene, a cationic polymer, can abolish completely the attachment inhibiting effect of human serum. It also prevents the clumping phenomenon caused by fresh clumping serum as well as by anionic polymers. The reason for this effect seems to be that the attachment of the cells to the glass surface is rapid and thus no clumping will occur (*Nordling et al.* 1964). If the glass surface was first treated with Polybrene its presence in the medium was no longer necessary, which indicates that it attaches to and alters the properties of the glass surface. This is supported by the fact that when the same Polybrene solution was changed from test tube to test tube the solution finally became ineffective. The attachment must be very strong as neither several washings with an alkaline washing solution nor treatment for several hours with cold chromic acid nor sodium hydroxyd could completely restore the original properties of the glass as regards cell attachment and growth behaviour. This may cause troubles in laboratories using Polybrene.

Furthermore it is shown that Polybrene in rather small concentrations is toxic to cells. In this connection it is of interest that cationic polymers like basic poly α-amino acids have been reported to be very toxic to animals, see e.g. *de Vries et al.* (1953). The impression that

A "Polybrene like" effect can be obtained by keeping the cells first in serum-free medium in the culture tubes, and then carefully pipetting on the serum In less than 10 minutes, the cells in a serum-free medium sediment and attach to the glass surface Aged serum pool as well as fresh non-clumping serum do not detach the already attached cells, whereas fresh clumping serum does so (*Nordling et al* 1964 b) This is of assistance in our comprehension of the reason why the cells are some distance apart in a Polybrene-containing medium As soon as they reach the glass surface of the tube, they attach, as they do in serum free medium, whereas in a serum containing medium without Polybrene they slide down the concave surface of the tube, and first attach after hours of incubation

In tubes which had contained Polybrene at a concentration of 10 µg/ml, and which had subsequently been washed three times by the routine method, the Polybrene effect was still detectable In this instance when Polybrene is not present in the medium, but only on the glass surface, the appearance of the cell culture is of a somewhat different type The cells are still far removed from each other, because of the rapid attachment, they are not large and round, but fibroblast-like, and it is impression that more cells have divided Not even by treating the tubes with 1 N NaOH or cold chromic acid, rather drastic methods, could the "Polybrene-effect" be made to disappear completely

If the same Polybrene solution of 6 µg/ml was changed from culture tube to culture tube, the solution finally became ineffective, indicating that all Polybrene had attached to the glass surface If cells were treated for 1 hour with 10 µg/ml Polybrene in a serum containing medium, they behaved like untreated cells In serumfree medium, however, it seems that some activity is adsorbed to the cell, or the cells are otherwise damaged

Agglutination Experiments

At a concentration of at least 4 µg/ml Polybrene causes the agglutination of red blood cells in a serum-free medium At higher concentrations the agglutination becomes stronger (Table 1) It was also observed that the red blood cells attached very strongly in a Polybrene containing buffered saline solution and that the shape of the red blood cells was completely changed when they attached to the glass surface, but this will be reported in a later paper (*Nordling* 1964) The agglutination of HeLa cells was difficult to evaluate as the trypsinized cells even in the absence of Polybrene formed small aggregates, but in the presence of 25 µg/ml Polybrene in a serumfree medium the aggregates were larger and more frequent The agglutination of mechanically dissociated HeLa cells was impossible to evaluate as many and even large clusters were present in the controls

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account the possibility that the effect of Polybrene is attributable to the possible phenomenon that less protein is adsorbed to a Polybrene treated glass surface than to an "ordinary" glass surface. This has already been suggested in the case of differently washed glass surfaces. It seems unlikely, however, that the difference in adsorption could be sufficiently great to explain the difference in attachment rate. An action mainly on the cell, provided by altering the surface charge of the cell as suggested by *Lieberman & Ove* (1958) in the case of salmine a protamine seems also most unlikely as the presence of the cationic polymer in the medium is not necessary. Furthermore protamine sulphate when present only in the medium has no or only a very small effect on the attachmeent of HeLa cells in a serum-containing medium (*Nordling* 1964). This, of course, does not exclude the possibility that Polybrene in some way alters the surface of the HeLa cell, but under the present experimental conditions the rôle of this possible effect is insignificant.

The effects of anionic polymers and Polybrene are opposite as regards cell attachment and growth behaviour. It is just possible that principally the same mechanisms are involved in both cases. This has not yet been proved, but studies on the matter are in progress.

SUMMARY

Polybrene, a quaternary ammonium polymer, greatly enhances the attachment of HeLa cells in a serum containing medium to a glass surface. When polybrene has acted on the glass surface its presence in the medium is no longer necessary. This indicates that it mainly acts on the glass surface and makes it positively charged and that the mechanism of attachment in this case is an attraction of particles of opposite charge.

Polybrene also inhibits the clumping effect of fresh clumping serum as well as of anionic polymers. This effect is probably due to the [illegible]

[illegible] strong alkali nor chromic acid for several hours could completely restore the original properties of the glass surface as regards cell attachment and growth behaviour.

The described effects seem to be a general feature of cationic polymers.

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Polybrene reduces the mitotic activity of HeLa cells is consistent with results obtained by *Richardsson et al* (1958) In a study on the effect of polylysine in malignant tumours in mice, they noted that the mitotic index decreased from 3 6 to 1 6

The reported effects of Polybrene seems to be a general feature of cationic polymers and macromolecules Protamine sulphate in a con centration of 30 μg/ml has an effect similar to that of Polybrene (*Nordling* 1964) *Lieberman & Ove* (1958) have reported that salmine, a basic protein, in a concentration of 8 μg/ml as well as polylysine enhanced the attachment of Appendix A 1 cells so that all cells were attached within some minutes According to these authors the probable mech in ism is that salmine attaches to the negatively charged cell and makes it positively charged, and thus more adhesive *Katchalsky et al* (1959) noted that red blood cells, which are very unsticky (*Bangham et al* 1958), *Curtis* 1962), in a medium containing cationic polymers like polylysine and protamine sulphate attached strongly to a glass surface Even by vigorous shaking, the cells could not be removed The same polymers also caused red blood cells to agglutinate They conclude that cationic polymers form links, not only between cells but also between the glass surface and the cell surface *Nevo et al* (1955) found that polylysine irreversibly attached to glass and could not be removed by ordinary washing but by 1 N NaOH The electrophoretic mobility of both glass and red blood cells were changed after polylysine treatment, polylysine also becomes adsorbed to the surface of red blood cells but this adsorption is reversible

The results obtained with red blood cells do not necessarily apply to HeLa cells The isoelectric point of red blood cells, if they exhibit a true isoelectric point at all, is in the order of 1 7 (*Abramsson* 1942, *Bangham et al* 1958, *Bangham & Pethica* 1960), whereas that of HeLa cells is much higher, about 4 (*Nordling & Hayry*) As the isoelectric point of HeLa cells is higher, the polymer is expected to be more weakly attached to the HeLa cell than to the red blood cell This may be an explanation why Polybrene causes a much stronger agglutination of red blood cells than of HeLa cells However, the evaluation of the agglutination of HeLa cells is difficult as non-trypsinized HeLa cells and to a lesser extent trypsinized ones in high concentrations tend to form clusters Furthermore trypsinization diminishes the electrical charge of cells (*Ponder* 1951, *Pondman & Malenbroek* 1954, *Seaman & Heard* 1960) The reversibility of the adsorption of cationic polymers to red cells probably also applies to HeLa cells This may explain why no distinct effect was obtainable by keeping HeLa cells first in a Polybrene containing me dium and then changing them into a Polybrene-free medium

The adhesion of HeLa cells to a Polybrene treated glass surface seems to be an electrostatic attraction of particles of opposite charge But the attachment inhibition of serum seems to be due to a serum film adsorbed to the glass surface (*Taylor* 1961) Thus one has to take into

account the possibility that the effect of Polybrene is attributable to the possible phenomenon that less protein is adsorbed to a Polybrene treated glass surface than to an ordinary glass surface. This has already been suggested in the case of differently washed glass surfaces. It seems unlikely, however, that the difference in adsorption could be sufficiently great to explain the difference in attachment rate. An action mainly on the cell provided by altering the surface charge of the cell as suggested by *Lieberman & Ove* (1958) in the case of salmine a protamine seems also most unlikely as the presence of the cationic polymer in the medium is not necessary. Furthermore protamine sulphate when present only in the medium has no or only a very small effect on the attachment of HeLa cells in a serum containing medium (*Nordling* 1964). This of course does not exclude the possibility that Polybrene in some way alters the surface of the HeLa cell but under the present experimental conditions the role of this possible effect is insignificant.

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MILIA FORMATION IN ORAL LESIONS IN EPIDERMOLYSIS BULLOSA

By

J O ANDREASEN E HJORTING HANSEN M ULMANSKY [1]
and J J PINDBORG

Received 27 vii 64

Epidermolysis bullosa is a rare condition affecting the skin and mucous membranes From a clinical point of view three main types are known simplex dystrophic and lethalis Epidermolysis bullosa simplex is a mild form of the disease in which mucous membrane involvement is rarely found In the dystrophic type bullae may be seen in the oral mucous membranes and changes in nails, hairs and teeth may appear (*Touraine* 1942, *Kazlick & Brustein* 1961)

In skin lesions of epidermolysis bullosa the occurrence of small epidermal cysts (milia) has been described A review of the literature shows that *Sakaguchi* (1916) found 35 out of 179 cases to be complicated with milia Their presence is not pathognomonic of epidermolysis bullosa as they may also appear in other dermatological conditions such as dermatitis herpetiformis pemphigus and lichen ruber planus (Sakaguchi 1916) The histiogenesis of milia in the skin in epidermolysis bullosa has been discussed for many years Four possible origins of milia in skin have been suggested

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MILIA FORMATION IN ORAL LESIONS IN EPIDERMOLYSIS BULLOSA

By

J. O. ANDREASEN, J. HJØRTING-HANSEN, M. ULMANSKY[1] and J. J. PINDBORG

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Epidermolysis bullosa is a rare condition affecting the skin and mucous membranes. From a clinical point of view three main types are known: simplex, dystrophic and lethalis. Epidermolysis bullosa simplex is a mild form of the disease in which mucous membrane involvement is rarely found. In the dystrophic type bullae may be seen in the oral mucous membranes, and changes in nails, hairs and teeth may appear (*Touraine* 1942, *Kaplick & Brustein* 1961).

In skin lesions of epidermolysis bullosa the occurrence of small epidermal cysts (milia) has been described. A review of the literature shows that *Sakaguchi* (1916) found 35 out of 179 cases to be complicated with milia. Their presence is not pathognomonic of epidermolysis bullosa, as they may also appear in other dermatological conditions such as dermatitis herpetiformis, pemphigus and lichen ruber planus (*Sakaguchi* 1916). The histiogenesis of milia in the skin in epidermolysis bullosa has been discussed for many years. Four possible origins of milia in skin have been suggested:

(1) They may be retention cysts of excretory ducts from underlying sweat glands (*Bukovsky* 1903, *Sakaguchi* 1916, *Love & Montgomery* 1943).

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PLATE II

PLATE I

Fig A Photograph of the fingers Note the atrophic scars and milia

Fig B Palatal nodules in areas of previous bullae formation

Fig C Bulla in posterior area of left cheek (arrow)

Fig D Histological section from one of the nodules in the palate (Fig B) Note the epithelial islands in the connective tissue and the flattening of the overlying epithelium Haematoxylin eosin stain × 40

PLATE II

Fig A — the cheek biopsy, presenting bulla and 125

Fig B el of the basement membrane Haema

Fig C — the connective tissue forming the base of × 250

Fig D of epithelial papillae close to a bulla

Fig E ea of early detachment Haematoxylin

Fig F Marked area from Figure A presenting two milia in different degrees of development Haematoxylin eosin stain × 40

Fig G High magnification of marked area from Figure F Note the peripheral flattening of the epithelial cells and the swelling pyknosis and vacuolization Haematoxylin eosin stain × 250



PLATE II

As milia in the oral cavity represent an unusual feature it is felt worthwhile to report a case of epidermolysis bullosa complicated with milia, both in the skin and in the oral mucosa

CASE REPORT

This study was made on biopsies from the oral mucosa of a 13 year old boy with epidermolysis bullosa dystrophica The disease could be followed in three generations on the male side whereas his two brothers were completely healthy

The patient presented typical skin lesions and milia (Plate I Fig A) An intraoral examination revealed elevated nodules in the hard palate (Pate I, Fig B), and two bullae in the posterior area of the left cheek (Plate I Fig C) one of the bullae was filled with a clear fluid whereas the other was filled with blood The teeth were of normal shapes and sizes and no enamel hypoplasias were found Biopsies were taken from the bulla in the cheek and from one of the palatal nodules After fixation in formalin the specimens were embedded in paraffin cut serially, and stained with haematoxylin eosin as well as a modified Mallory stain (*Weinmann & Meyer* 1959)

Microscopical examination of the specimens of the cheek revealed that the epithelium was of normal thickness (Plate II Fig A) Bullae in different stages of development were observed the bigger ones of these were filled with eosinophilic fluid The onset of bulla formation was seen in the subepithelial region and generally localized to the tips of the rete pegs (Plate II Fig B) Often islands of epithelial remnants could be seen in the connective tissue forming the base of a bulla or close to the epithelium (Plate II Fig C) The serial sections revealed that they were true epithelial islands and not tangential cuts of rete pegs Epithelial proliferations from the periphery were seen along the base of the bulla In some places parts of rete pegs were partly separated from the rest of the epithelium (Plate I Fig D and Plate II Fig D E) Lamina propria showed increased vascularity with dilated capillaries and in relation to the bullae a moderate chronic non specific inflammation Immediately under the epithelium were three small cysts (milia) (Plate II Fig A and F) In that area were no rete pegs but except for this the overlying epithelium was of normal thickness The periphery of the cysts consisted of 2 3 layers of flattened epithelial cells Centrally disruption of the intercellular bridges and epithelial cells with pyknotic nuclei was noted The cells were partly eosinophilic and stained like keratin with the modified Mallory stain (Plate II Fig G)

DISCUSSION

Examination of the histological sections showed several islands of epithelium in the connective tissue It was also noted that part of the rete pegs were becoming detached from the rest of the epithelium and apparently, after complete separation, remained close to their origin in the lamina propria These epithelial elements may be responsible for the milia formation In our specimens from the cheek, milia were intermingled with epithelial islands in different degrees of transformation This change into milia can be regarded as a result of a possible peripheral growth of these islands with a corresponding swelling of the cells, eosinophilia and pyknosis Previous studies on the pathogenesis of milia in the skin claim that their origin is concerned with the obstruction of excretory ducts of underlying sweat glands (*Bukovsky* 1903, *Sakaguchi* 1916, *Love & Montgomery* 1943) or hair follicles (*Epstein & Kligman* 1956) The milia found in our case must have another pathogenesis as sweat glands and hair follicles were not found in the oral mucosa sebaceous glands were not present in our

specimen A small accumulation of mucous gland acini was seen in the deepest part of the biopsy from the cheek Any relation between this area and the milia was not seen in any of the sections For this reason the milia in the present case were formed, apparently, from islands of epithelium detached during the bulla formation This pathogenesis is in accordance with the statements of *Adamson* (1905), and *Epstein & Kligman* (1956), regarding the formation of milia in skin

SUMMARY

A study was made on biopsy material from oral lesions in a case of epidermolysis bullosa dystrophica The presence of milia in the oral cavity was reported Apparently, the milia were not retention cysts but originated from detached islands of epithelium in areas of earlier bulla formation

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Microscopical examination of the specimens of the cheek revealed that the epithelium was of normal thickness (Plate II, Fig. A). Bullae in different stages of development were observed, *the bigger ones of these were filled with eosinophilic* fluid. The onset of bulla formation was seen in the subepithelial region and generally localized to the tips of the rete pegs (Plate II, Fig. B). Often islands of epithelial remnants could be seen in the connective tissue forming the base of a bulla or close to the epithelium (Plate II, Fig. C). The serial sections revealed that they were true epithelial islands and not tangential cuts of rete pegs. Epithelial proliferations from the periphery were seen along the base of the bulla. In some places parts of rete pegs were partly separated from the rest of the epithelium (Plate I, Fig. D and Plate II, Fig. D, E). Lamina propria showed increased vascularity with dilated capillaries and in relation to the bullae a moderate chronic non specific inflammation. Immediately under the epithelium were three small cysts (milia) (Plate II, Fig. A and F). In that area were no rete pegs but except for this the overlying epithelium was of normal thickness. The periphery of the cysts consisted of 2-3 layers of flattened epithelial cells. Centrally disruption of the intercellular bridges and epithelial cells with pyknotic nuclei was noted. The cells were partly eosinophilic and stained like keratin with the modified Mallory stain (Plate II, Fig. G).

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Regularly, DNA exposure was allowed to proceed for 15 min In some supplementary experiments however, the recipient in question was exposed continuously to DNA during the phenotypic expression period These experiments were not considered quantitative but semiquantitative (*Bövre* 1964b)

As a rule, transformants were scored at the concentration of 500 µg streptomycin per ml In heterologous transformation of *Moraxella nonliquefaciens* 4663 62 with *Moraxella bovis* 10900 DNA the selection concentrations 10 50, 100 500 and 1000 µg per ml were applied in parallel

As before donor strains and transformants were controlled for uniformly high streptomycin resistance by simple surface streaking and by replica plating

A supplementary methodological study was performed to clarify whether competition between transformants and streptomycin sensitive recipient cells was of importance for th

...unless untransformed bacteria were added to give the same ratio of sensitive cells to transformants as in the less diluted heterologous system

In intrastrain transformation of *Moraxella nonliquefaciens* 7784 the following experiment

... exposed cells The expression period was 9 h Both the inoculum size and the expression period in this experiment were at the extreme of the procedure generally appl...

... where heterologous transformants ... computed in undiluted and tenfold diluted parallels scores were approximately proportionate Consequently, there is no indication that dilution is critical at these relatively high ratios of sensitive cells to transformants

... with somewhat prolonged expression periods are included in some of the experiments for control of maximal phenotypic expression

RESULTS

Description of the Strains of Moraxella bovis

The microscopical picture was roughly in accordance with that of *Moraxella nonliquefaciens* (*Bovre* 1964b), although plumpness was less pronounced in two strains The occurrence of very short diplobacilli or diplococci seemed to be a constant characteristic of the other strains (Figs 1 and 2) Resistance to decolorization during Gram-staining was characteristic

The colonies regularly reached 1 5 mm in diameter after 20 h incubation on blood agar, and thus the colonies were a little greater than

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STUDIES ON TRANSFORMATION IN MORAXELLA AND ORGANISMS ASSUMED TO BE RELATED TO MORAXELLA

3 *Quantitative Streptomycin Resistance Transformation between* Moraxella bovis *and* Moraxella nonliquefaciens *Strains*

By

Kjell Bøvre

Received 11 vi 64

In previous communications transformation of *Moraxella bovis* with homologous DNA and transformation between *Moraxella bovis* and *Moraxella nonliquefaciens* have been briefly reported or referred to (*Bøvre & Henriksen* 1962, *Bøvre* 1963, *Bøvre* 1964a) It is the intention here to present quantitative streptomycin resistance transformation reactions among *Moraxella bovis* strains, and reciprocal transformation reactions between *Moraxella bovis* and *Moraxella nonliquefaciens*, based on representative strains of the two assumed species (see *Bøvre* 1964b)

MATERIAL AND METHODS

4 type culture collection strains of *Moraxella bovis* were included 3 of which had been received from the National Collection of Type Cultures London (strains 9425 9426 and 8561) and 1 strain from the American Type Culture Collection (strain 10900)

The *Moraxella bovis* strains were examined morphologically culturally and biochemically according to the same conventional methods and with the same media as described for the examination of *Moraxella nonliquefaciens* (*Bøvre* 1964b) The [illegible] l Agar Base (Difco) with 5 per [illegible] s were performed as described [illegible] the present study have all been [illegible] and quantitative interstrain transformation (loc cit)

The methods of mutant selection DNA extraction and quantitative streptomycin resistance transformation were in accordance with those reported (*Bøvre* 1964a) Controls of DNA saturation and effectivity of termination of DNA exposure by means of DNase were performed frequently but were not considered necessary in all experiments In addition to controls with DNase treated DNA parallels with untreated recipient cells and with DNA extracted from sensitive cells were always negative thereby excluding spontaneous and induced mutation simulating transformation

At least 2 different DNA extracts from each *Moraxella bovis* donor were applied in experiments with the recipient *Moraxella nonliquefaciens* 7784 Saturation was always obtained with the usual 20 µg of total DNA per ml as measured with the method of Dische

TABLE 1

Characteristics of the Moraxella bovis Strains

...in	Microscopical picture	Consistency of colonies	Agglutinability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth in citrate	Nitrite production	Urease activity	Haemolysis	Serum liquefaction
...00	Almost slender rods*	Almost soft	—	+*	—	—**	—	+	++‖
...25	Predominantly coccoid chains	Soft	—	+	—	—	—	+	++
...126	Predominantly coccoid chains†	Soft	—	+	—	—	—	+	++
...561	Almost slender rods	Intermediate§	(+)	+	—	—	—	+	++

Fig 1 † See Fig 2 § Intermediate between soft and friable * + = Feeble growth
** = Nitrate apparently not attacked as shown by means of Zn powder reduction
‖ + = Rapid reaction clearly evident in 20 h

TABLE 2

Sensitivity to Antibiotics of the Moraxella bovis Strains

Antibiotic	Range of inhibition zones in mm	Approximate range of minimum inhibitory concentration (m i c)*
Penicillin	32 38	0 09 0 02 I U/ml
Streptomycin	27 32	0 2 –0 02 µg/ml
Chloramphenicol	30 43	0 07 0 03 µg/ml
Oxytetracycline	33 37	0 03–0 008 µg/ml
Erythromycin	33 38	0 2 –0 04 µg/ml

* M i c values were calculated from zone diameters by means of regression equations (*Ericsson* 1960)

...otics (Table 2) Other characteristics are collected in Table 1

Transformation Reactions

The ratios of interstrain to intrastrain transformation frequencies within *Moraxella bovis* are demonstrated in Table 3 It is evident that all strains show a rough uniformity in terms of quantitative streptomycin resistance transformation, the ratios of inter- to intrastrain transformation ranging from 5 3 10^{-1} to 1 1 10^{0}

In Table 4 are presented quantitative transformation experiments between several *Moraxella nonliquefaciens* donors and the recipient strain *Moraxella bovis* 10900 6 of the donors belong to the intertrans-

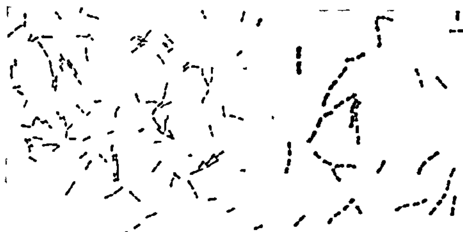


Fig 1 Fig 2

Fig 1 Moraxella bovis 10900 stained with Löffler's methylene blue × 1000
Fig 2 Moraxella bovis 9426 stained with Löfflers methylene blue × 1000

the average colonies of *Moraxella nonliquefaciens* under the same conditions (loc cit) The colonies were low conical or flat, round, even and glistening, but often with a rough appearance when subcultured from a less viable culture They were nonpigmented, but generally with a more pronounced opacity than *Moraxella nonliquefaciens* colonies Nevertheless, they gave a delicate impression and the opacity seemed to be arranged in a fine, irregular network when observed in transmitted light

All strains yielded a broad, completely haemolytic zone around the colonies, 3 to 4 mm in diameter after 20 h incubation

The strains grew almost as well at 32 33° C as at 37° C, with a slight preference for the latter temperature, contrary to many *Moraxella nonliquefaciens* strains On blood agar, a humid atmosphere improved growth considerably All strains failed to grow anaerobically In 0 4 per cent Brain Heart Infusion agar stab culture all strains grew fairly well down to 5 mm below the surface In fluid media growth was very poor and irregular The strains 9425 and 9426 were unable to grow in the medium for nitrite production The strains 10900 and 9425 did not grow in the indol medium described, whereas growth was feeble in the instances not mentioned No strain grew in the medium for hydrogen sulphide production The biochemical reactions in question were retested with very heavy inocula Nevertheless, negative reactions noted under these circumstances may be questioned The strains all grew well on glucose ascites agar and on coagulated bovine serum On the surface of Hugh & Leifson's medium feeble growth was observed in contrast to *Moraxella nonliquefaciens* (loc cit) (The test for growth on the surface of Hugh & Leifson's medium seems to parallel very closely the ability to grow in ordinary 1 per cent peptone water)

The strains were all Gram-negative, immotile and oxidase positive, and did not produce acid from glucose (tested on glucose ascites agar

TABLE 1

Characteristics of the Moraxella bovis Strains

Strain	Microscopical picture	Consistency of colonies	Agglutinability in physiological saline	Growth on the surface of Hugh & Leifson's medium	Growth in citrate	Nitrite production	Urease activity	Haemolysis	Serum liquefaction‖
10900	Almost slender rods*	Almost soft	—	+•	—	—••	—	+	++
9425	Predominantly coccoid chains	Soft	—	+	—	—	—	+	++
9426	Predominantly coccoid chains†	Soft	—	+	—	—	—	+	++
8561	Almost slender rods	Intermediate§	(+)	+	—	—	—	+	++

See Fig 1 † See Fig 2 § Intermediate between soft and friable • + = Feeble growth
•• — = Nitrate apparently not attacked as shown by means of Zn powder reduction
‖ ++ = Rapid reaction clearly evident in 20 h

TABLE 2

Sensitivity to Antibiotics of the Moraxella bovis Strains

Antibiotic	Range of inhibition zones in mm	Approximate range of minimum inhibitory concentration (m i c)*
Penicillin	32 38	0 09 0 02 I U/ml
Streptomycin	27 32	0 2 –0 02 µg/ml
Chloramphenicol	39 43	0 07 0 03 µg/ml
Oxytetracycline	33 37	0 03 0 008 µg/ml
Erythromycin	33 38	0 2 0 04 µg/ml

* M i c values were calculated from zone diameters by means of regression equations (*Ericsson* 1960)

(Table 2) Other characteristics are collected in Table 1

Transformation Reactions

The ratios of interstrain to intrastrain transformation frequencies within *Moraxella bovis* are demonstrated in Table 3 It is evident that all strains show a *rough uniformity* in terms of quantitative streptomycin resistance transformation, the ratios of inter- to intrastrain transformation ranging from 5 3 10 1 to 1 1 10⁰

In Table 4 are presented quantitative transformation experiments between several *Moraxella nonliquefaciens* donors and the recipient strain *Moraxella bovis* 10900 6 of the donors belong to the intertrans-

formable group of *Moraxella nonliquefaciens*, whereas the strains 19116/51 and 752/52 have been found to deviate from that group in terms of transformation and according to conventional criteria (*Bovre* 1964b) The ratios of inter- to intrastrain transformation between the representative *Moraxella nonliquefaciens* strains and *Moraxella bovis* are ranging from 1 7 10^{3} to 2 6 10^{3} The strains which deviate from the intertransformable *Moraxella nonliquefaciens* strains are also deviating from *Moraxella bovis*

TABLE 3

Ratios of Interstrain to Intrastrain Transformation to Streptomycin Resistance among Moraxella bovis *Strains Recipient* Moraxella bovis *10900*

Donor strain	Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml*	Ratio of inter to intrastrain transformation
M bovis 9425	5 0 10^{7}	7 6 10^{2} (76)§	7 0 10- (70)	1 1 10^{0}
M bovis 9425	1 0 10^{8}	2 3 10^{3} (234)	2 4 10^{3} (241)	9 7 10^{1}
M bovis 9426	5 0 10^{7}	4 2 10^{2} (42)	7 0 10' (70)	6 0 10^{1}
M bovis 9426	1 0 10^{8}	1 3 10^{3} (128)	2 4 10^{3} (241)	5 3 10^{1}
M bovis 8561	5 0 10^{7}	4 6 10- (46)	7 0 10 (70)	6 6 10^{1}
M bovis 8561	1 0 10^{8}	1 3 10^{3} (130)	2 4 10^{3} (241)	5 4 10^{-1}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml

* Intrastrain transformants were scored in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 2 or 3 plate counts

TABLE 4

Transformation of Moraxella bovis *to Streptomycin Resistance by DNAs from* Moraxella nonliquefaciens *Strains Recipient* Moraxella bovis *10900*

Donor strain	Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml*	Ratio of inter- to intrastrain transformation
M nonliq 2770/60	7 6 10	1 0 10^{2} (10)§	4 6 10^{4} (46)	2 2 10^{3}
M nonliq 7784		1 2 10- (12)		2 6 10^{-3}
M nonliq 3828/60		1 1 10 (11)		2 4 10^{3}
M nonliq 178/62		1 2 10- (12)		2 6 10^{3}
M nonliq 820/61		9 0 10^{1} (9)		2 0 10^{3}
M nonliq 1962/62		8 0 10^{1} (8)		1 7 10^{3}
Strain 19116/51†		<10^{1} (0)°		<2 2 10^{4}
Strain 752/52†	not counted	<10^{1} (0)°	3 0 10^{3} (30)	<3 3 10^{3}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml

* Intrastrain transformants were scored in simultaneous transformation of the recipient with its own mutant DNA

§ [illegible] 3 plate counts

† [illegible] terms of transformation from most Morax [illegible]

° In parallel [illegible] transf rmants were still not detected

TABLE 5

Transformation of Moraxella nonliquefaciens *to Streptomycin Resistance by DNAs from* Moraxella bovis *Strains*

Recipient strain of M nonliq	Donor strain of M bovis	Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml[*]	Ratio of inter to intrastrain transformation
4663 62	10900	8 0 10^{7}	3 0 10^{2} (30)§	1 5 10^{5} (38)	2 0 10^{-3}
	9425	2 5 10	3 9 10^{2} (39)	1 9 10^{5} (97)	2 0 10^{-3}
	9426	2 5 10	3 6 10^{2} (36)	1 9 10^{5} (97)	1 9 10^{-3}
	8561	2 5 10^{7}	3 6 10^{2} (36)	1 9 10^{5} (97)	1 9 10^{-3}
7784	10900	1 0 10^{7}	1 1 10^{2} (11)	6 0 10^{4} (60)	1 8 10^{-3}
		1 0 10^{8}	4 3 10^{2} (43)	2 3 10^{5} (58)	1 5 10^{-3}
	"	1 1 10^{7}	4 4 10^{2} (44)	2 5 10^{5} (246)	1 8 10^{-3}
	"	3 0 10^{6}	2 0 10^{2} (20)	1 2 10^{5} (118)	1 7 10^{-3}
	9425	1 0 10^{8}	1 5 10^{3} (146)	2 3 10^{5} (58)	5 0 10^{-3}
		1 1 10	1 5 10^{3} (147)	2 5 10^{5} (246)	6 0 10^{-3}
	"	3 0 10^{6}	8 9 10^{2} (89)	1 2 10^{5} (118)	7 5 10^{-3}
	9426	1 0 10^{8}	5 3 10^{2} (53)	2 9 10^{5} (58)	1 8 10^{-3}
		1 1 10	5 5 10^{2} (55)	2 5 10^{5} (246)	2.2 10^{-3}
	8561	1 0 10^{8}	4 7 10^{2} (47)	2 3 10^{5} (58)	1 6 10^{-3}
		1 1 10	4 2 10^{2} (42)	2 5 10^{5} (246)	1 7 10^{-3}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml The lower part of the table shows results obtained with 2 or more DNA extracts from each donor

[*] Intrastrain transformants were scored in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 6 plate counts in the upper part of the table and generally means of 3 plate counts in the lower part

Strain 19116.51 was also used as recipient in quantitative transformation with *Moraxella bovis* 10900 as the donor In that case the transformation ratio was found to be less than 1 0 10^{-3} (not tabulated) In the experiments with strains 19116.51 and 752.52 no transformants were detected with continuous DNA exposure either, which means that the reactions with *Moraxella bovis* of strain 19116 51 and probably also of strain 752.52 are far more infrequent than the representative *Moraxella nonliquefaciens Moraxella bovis* ratios

Table 5

don

466 ... the ratios of inter to intrastrain transformation frequencies are here ranging from 1 5 10^{-3} to 7 5 10^{-3}, with the majority of results from 1 5 10^{-3} to 2 0 10^{-3} The more

7784 with *Morax*

was very active in

... of *Moraxella bovis* 10900 ...

... nonspecific quality of the DNA

formable group of *Moraxella nonliquefaciens*, whereas the strains 19116/51 and 752/52 have been found to deviate from that group in terms of transformation and according to conventional criteria (*Bovre* 1964b) The ratios of inter- to intrastrain transformation between the representative *Moraxella nonliquefaciens* strains and *Moraxella bovis* are ranging from 1 7 10 3 to 2 6 10 3 The strains which deviate from the intertransformable *Moraxella nonliquefaciens* strains are also deviating from *Moraxella bovis*

TABLE 3

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Donor strain	Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml*	Ratio of inter to intrastrain transformation
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Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml

* Intrastrain transformants were scored in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 2 or 3 plate counts

TABLE 4

Transformation of Moraxella bovis *to Streptomycin Resistance by DNAs from* Moraxella nonliquefaciens *Strains Recipient* Moraxella bovis *10900*

Donor strain	Recipient count ml	Interstrain transformants ml	Intrastrain transformants ml*	Ratio of inter- to intrastrain transformation
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M nonliq 7784		1 2 10^{1} (12)		2 6 10^{-3}
M nonliq 3828/60		1 1 10^{1} (11)		2 4 10^{-3}
M nonliq 178/62		1 2 10^{1} (12)		2 6 10^{-3}
M nonliq 826/61		9 0 10^{1} (9)		2 0 10^{3}
M nonliq 1962/62		8 0 10^{1} (8)		1 7 10^{3}
Strain 19116/51†		$<10^{1}$ (0)°		$<$2 2 10^{-4}
Strain 752/52†	not counted	$<10^{1}$ (0)°	3 0 10^{3} (30)	$<$3 3 10^{3}

Duration of DNA exposure 15 min Selection concentration 500 µg streptomycin per ml

* Intrastrain transformants were scored in simultaneous transformation of the recipient with its own mutant DNA

§ Figures in brackets indicate means of 3 plate counts

† Strains previously found to deviate in terms of transformation from most *Moraxella nonliquefaciens* strains

° In parallel continuous DNA exposure transformants were still not detected

tion frequency is due to imperfect pairing of DNA structures (*Schaeffer* 1958)

Previously, replica plating of 100 colonies of the donor *Moraxella nonliquefaciens* 7784 without exception has revealed growth at 1000 µg streptomycin per ml (*Bøvre* 1964b) The same results were obtained with the donors *Moraxella bovis* 10900, 9125 and 9126 The remaining donors were all able to grow at this concentration when simply streaked on the surface of blood agar Streptomycin dependence was excluded

Although mutants selected according to the procedure described are expected to be single-step mutants, the possibility exists that mutants growing at 500 µg or higher concentrations of streptomycin per ml, have undergone more than one mutational step to reach this level of resistance As *Hotchkiss* (1952) first reported, the transforming ability of streptomycin resistant donor strains repeat the mutational history of those strains The usual selection concentration might therefore be critical for some of the transformants

That 500 µg of streptomycin per ml is not critical for intrastrain transformants in *Moraxella nonliquefaciens* 7784 and 4663 62, has been shown previously (*Bøvre* 1964b) In the transformation of *Moraxella nonliquefaciens* 4663 62 by means of *Moraxella bovis* 10900 DNA no differences were observed in the counts of transformants with selection concentrations varying from 10 to 1000 µg streptomycin per ml The transformants were all resistant to 1000 µg streptomycin per ml Also in other cases examined the transformants invariably had this high degree of streptomycin resistance, as shown in replica plating controls

Except for the transferred streptomycin resistance character, repeated examinations never entailed any deviation of phenotype of transformants, as compared with recipient cultures

DISCUSSION AND CONCLUSION

The *Moraxella bovis* strains included in this investigation are all type culture collection strains which share distinguishing characters listed for this species (*Murray* 1957) The differences observed in morphological and cultural properties are small and do not indicate any principal heterogeneity among the strains

The *Moraxella bovis* strains mainly differ from the intertransformable group of *Moraxella nonliquefaciens* (*Bøvre* 1964b) in their haemolytic activity serum liquefaction, absence of nitrite production from nitrate and in their growth ability on Hugh & Leifson's medium, as far as can be deduced from the few tests performed

The ratios of inter- to intrastrain transformation among the *Moraxella bovis* strains show that they are all within the limits suggested for a first degree relationship in terms of streptomycin resistance transformation in a previous discussion (loc cit)

The general reciprocal streptomycin resistance transformation ratio

TABLE 6

The Effect on Transformation Ratios by the Integration of Heterologous Streptomycin Resistance Marker in Donor DNA

Recipient strain	Recipient count ml	Donor DNA	Transformants ml
Moraxella nonliquefaciens 7784	5 0 10^{7}	(7784 Smr)	3 5 10[illegible]
		(7784 Smr 10900)	3 3 10[illegible]
Moraxella bovis 10900	1 0 10^{8}	(7784 Smr)	3 0 10[illegible]
		(7784 Smr 10900)	2 0 10[illegible]
Moraxella bovis 10900	1 8 10^{8}	(7784 Smr)	3 0 10^{1}
		(7784 Smr 10900)	2 1 10^{2}
Moraxella nonliquefaciens 7784	8 0 10	(10900 Smr 10900)	1 4 10[illegible]
		(10900 Smr 7784)	8 7 10[illegible]

(7784 Smr) = DNA of streptomycin resistant mutant of *Moraxella nonliquefaciens* 7784
(7784 Smr 10900) = DNA of the same strain after transformation to streptomycin resistance by *Moraxella bovis* 10900
(10900 Smr 10900) = DNA of *Moraxella bovis* 10900 which has been transformed to streptomycin resistance in intrastrain transformation
(10900 Smr 7784) = DNA of the latter strain after transformation to streptomycin resistance by *Moraxella nonliquefaciens* 7784

Table 6 represents an experiment which was undertaken to exclude that the low frequency of transformation between *Moraxella nonliquefaciens* and *Moraxella bovis* is due to the heterologous origin of the genetic marker itself In the upper part of the table it can be seen that following transformation of *Moraxella nonliquefaciens* with DNA from *Moraxella bovis,* DNA extracted from the transformant will practically behave as *Moraxella nonliquefaciens* DNA in transformation of the *Moraxella nonliquefaciens* recipient That the DNA of *Moraxella nonliquefaciens* transformed by *Moraxella bovis* has retained *some* of the *Moraxella bovis* specific structures is suggested in the middle part of the table, where it can be seen that *Moraxella bovis* is transformed about 7 times more frequently by *Moraxella nonliquefaciens* supplied with the marker in transformation by *Moraxella bovis* as compared with the mutant DNA of *Moraxella nonliquefaciens* That this phenomenon is reciprocal between *Moraxella bovis* and *Moraxella nonliquefaciens,* is shown in the lower part of the table

Findings very similar to those presented in Table 6 have previously been made by *Leidy, Hahn & Alexander* (1956) and later by *Schaeffer* (1958) in transformation between *Haemophilus influenzae* and *Haemophilus parainfluenzae* The results suggest that the low frequency of heterologous transformation is due to the heterologous origin of the DNA molecule as a whole, and not mainly to heterologies of the streptomycin resistance determinant itself The observed facts are considered consistent with the hypothesis that the lower heterologous transforma-

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THE INFLUENCE OF INTERFERON AND HYDROCORTISONE ON THE PRODUCTION OF INCOMPLETE INFLUENZA VIRUS IN OVO

By

V REINICKE and F V HAHNEMANN

Received 11 xi 64

It has been shown by *Hahnemann & Reinicke* (2) that in embryonated eggs inoculated with influenza virus of varying degree of incompleteness the production of interferon decreased with increasing incompleteness of the inoculum The possible rôle of interferon for the production of incomplete influenza virus was, however, not clearly elucidated by these investigations It has been suggested by *Gresser* (1) that production of incomplete influenza virus might be due to the effect of interferon on the virus producing cells This hypothesis was based on observations by this author and by *Isaacs et al* (4), which indicated that interferon inhibited the production of viral subunits in the cell nucleus to a greater extent than in the cytoplasm Since it has been shown that the infectious part of the influenza virus particle is synthesized in the nucleus of the cell (3 7) this effect of interferon might consequently be responsible for the formation of virus with a reduced infectivity/haemagglutinin (LID50 HA) ratio, i e incomplete virus

In order to investigate this hypothesis, a series of experiments has been carried out to study the effect of addition of interferon on th[illegible] of influenza virus under circumstances normally leading to the formation of incomplete virus

MATERIALS AND METHODS

Most material and methods have been described in detail in a previous paper (9)

Virus Stocks of influenza B Lee and influenza A Melbourne were employed These viruses were in the 7th to 9th egg passage in this laboratory and contained $10^{9.0}$–$10^{9.4}$ EID50 per ml Influenza B Lee virus was passaged immediately prior to each experiment and used as freshly harvested allantoic fluid

The authors wish to thank Mrs A Diemer and Mrs B Saugbjerg for skilled technical assistance

between *Moraxella bovis* and *Moraxella nonliquefaciens* is safely based on a controlled methodology and on representative strains, although the number of *Moraxella bovis* strains studied is rather small It is considered a well established quantitative genetic approach to the taxonomy of these bacteria However, its validity as a measure of overall genetic compatibility needs some confirmative investigations Also, the mapping of genetic relationships between these and other bacteria is developing and a full discussion will be more fruitful when more similar studies have been performed with other assumed species and groups

SUMMARY

4 type culture collection strains classified as *Moraxella bovis*, were investigated by means of conventional diagnostic methods and quantitative streptomycin resistance transformation All strains behaved as closely related strains, the ratios of inter to intrastrain transformation being within the limits previously suggested for a first degree relationship in terms of streptomycin resistance transformation

Reciprocal inter- to intrastrain ratios of streptomycin resistance transformation frequencies between the *Moraxella bovis* strains and representative *Moraxella nonliquefaciens* strains were determined The ratios ranged from 1 5 10^{3} to 7 5 10^{3}, with most observations close to the former value

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It has been shown by *Hahnemann & Reinicke* (2) that in embryonated eggs inoculated with influenza virus of varying degree of incompleteness the production of interferon decreased with increasing incompleteness of the inoculum The possible rôle of interferon for the production of incomplete influenza virus was, however, not clearly elucidated by these investigations It has been suggested by *Gresser* (1) that production of incomplete influenza virus might be due to the effect of interferon on the virus producing cells This hypothesis was based on observations by this author and by *Isaacs et al* (4), which indicated that interferon inhibited the production of viral subunits in the cell nucleus to a greater extent than in the cytoplasm Since it has been shown that the infectious part of the influenza virus particle is synthesized in the nucleus of the cell (3, 7) this effect of interferon might consequently be responsible for the formation of virus with a reduced infectivity/haemagglutinin (EID50 HA) ratio, i e incomplete virus

In order to investigate this hypothesis, a series of experiments has been carried out to study the effect of addition of interferon on the in ovo synthesis of influenza virus under circumstances normally leading to the formation of complete virus The observation of the inhibiting effect of cortisone and certain other steroids on the production of interferon in ovo (6 9) inspired to a further series of experiments in which an attempt was made by inhibiting the production of interferon in ovo, to affect the synthesis of influenza virus under circumstances normally leading to the formation of incomplete virus

MATERIALS AND METHODS

[illegible]

[illegible] us was passaged immediately prior to each experiment and used as freshly harvested allantoic fluid

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between *Moraxella bovis* and *Moraxella nonliquefaciens* is safely based on a controlled methodology and on representative strains, although the number of *Moraxella bovis* strains studied is rather small. It is considered a well established quantitative genetic approach to the taxonomy of these bacteria. However, its validity as a measure of overall genetic compatibility needs some confirmative investigations. Also, the mapping of genetic relationships between these and other bacteria is developing and a full discussion will be more fruitful when more similar studies have been performed with other assumed species and groups.

SUMMARY

4 type culture collection strains classified as *Moraxella bovis*, were investigated by means of conventional diagnostic methods and quantitative streptomycin resistance transformation. All strains behaved as closely related strains, the ratios of inter- to intrastrain transformation being within the limits previously suggested for a first degree relationship in terms of streptomycin resistance transformation.

Reciprocal inter- to intrastrain ratios of streptomycin resistance transformation frequencies between the *Moraxella bovis* strains and representative *Moraxella nonliquefaciens* strains were determined. The ratios ranged from 1.5×10^{-3} to 7.5×10^{-3}, with most observations close to the former value.

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TABLE 1

Virus Titres and Infectivity/HA Ratios in Allantoic Fluids from Eggs Having Received a Dose of Interferon at Varying Intervals of Time before and after the Inoculation of $10^{3.4}$ EID50 of Influenza B Lee Virus

Inoculated with	Titres and ratios	Interferon inoculated at hours before and after the virus inoculation						
		−12	−6	−3	0	+3	+12	+24
Interferon	HA	<0 30*	<0 30	<0 30	<0 30	<0 30	<0 30	2 66
	EID50	5 4	5 0	6 0	6 6	5 6	6 6	8 2
	EID50/HA	>5 10	>4 70	>5 70	>6 30	>5 30	>6 30	5 54
Control	HA	3 18	3 18	3 11	3 03	3 11	3 11	3 11
	EID50	9 4	9.2	9 4	9 0	8 8	9 0	9.2
	EID50/HA	6 22	6 02	6 29	5 97	5 69	6 79	6 09

* All titres are recorded as $\log_{10}$

hibiting effect on the HA titres as well as on the infectivity titres whether the interferon had been inoculated 12, 6 or 3 hours *before* the virus inoculation Inoculation of interferon immediately after the virus inoculation as well as 3 and 12 hours later also caused a marked inhibition of the synthesis of virus, though slightly less than when interferon was administered before the inoculation of virus The group *inoculated with interferon 24 hours after* the virus inoculation shows only a moderate degree of inhibition

The infectivity/HA ratio of the seven control groups varied from 5 69 to 6 79 indicating that all these *fluids as expected contained* complete virus Of the seven interferon treated groups, only the group treated 24 hours after the virus inoculation produced sufficient amounts of haemagglutinin to allow a calculation of the infectivity/HA ratio The *ratio* of the pooled fluids from this group was 5 54 Although slightly lower than in the *controls this ratio* indicates that the yield consisted of predominantly fully active virus In the remaining 6 interferon treated groups the infectivity/HA ratios could not be calculated with certainty since the content of haemagglutinin was below measurable levels However, the fluids harvested from the eggs ...

... field virus

In order to obtain a less pronounced *inhibition* of the synthesis of haemagglutinin and hereby a more reliable estimate of the infectivity/HA ratio in interferon treated eggs another experiment ...

... ml of undiluted interferon at varying times before and after the virus inoculation (see Table 2) Seven other groups were given 1 ml of undiluted *interferon* at the same time inter-

Eggs 10 to 11 days embryonated white Leghorn eggs were employed

Titrations of virus Infectivity titres and haemagglutinin (HA) titres were measured as previously described (9).

Hydrocortisone Di (17 hydroxycorticosterone-21) phosphoriacidester sodium salt (Actocortin) dissolved in distilled water was employed A dose of 250 γ of di (17 hydroxycorticosterone-21) phosphoriacidester sodium salt was used since such a dose previously has been shown to have a marked inhibiting effect on the in ovo synthesis of interferon following inoculation of approximately $10^{3.5}$ EID50 of influenza B Lee virus (9)

Dialysis was performed against distilled water and phosphate buffer at pH = 7 38

Interferon was prepared by allantoic inoculation of 10 day-old embryonated eggs with $10^{3.5}$ EID50 of influenza B Lee virus After 72 hours of incubation the allantoic fluids were harvested and pooled and the virus present in the pool was inactivated by heating to 65° C for one hour After it had been ascertained that the inactivated pool was non-infectious and without measurable amounts of haemagglutinin, the pool was centrifuged at low speed and subsequently spun in a spinco Model L centrifuge for one hour at 30 000 r p m (105 000 g) followed by one hour at 40 000 r p m (144 000 g) The supernatant from the last of the two high speed centrifugations was used as the batch of interferon The titre of the batch was measured as described earlier (9) and found to be $10^{2.1}$ A batch of control fluid from non inoculated 13 day old eggs was prepared in the same way This control fluid had no interfering activity

In the experiments with hydrocortisone the content of interferon in heat-inactivated dialysed pools of allantoic fluid was measured by a modification of the method described earlier (9) Briefly, the method depends upon incubation of chorio [illegible] followed by

tinin 48

control

le's BSS

before the challenge) allowed an estimation of the content of interferon The titre of interferon was expressed as the difference

$$\text{HA titre of the control (100 per cent)} \quad \frac{\text{HA titre of the sample} \times 100}{\text{HA titre of the control}} \%$$

Antibiotics G penicillin and streptomycin giving a final concentration of 200 units/ml and 50 γ/ml, respectively, were added to the batch of interferon to the buffer and to all pools of allantoic fluids

EXPERIMENTAL

In order to investigate the effect of addition of interferon on the degree of completeness of influenza virus produced in ovo, the following experiment was performed 14 groups of 10-day-old embryonated eggs, each group consisting of 10 eggs were inoculated by the allantoic route with influenza B Lee virus, each egg receiving a dose of $10^{3.4}$ EID50 The eggs were all harvested after incubation for 44 hours at 36° C Under such circumstances the virus yield normally consists of complete virus Seven of the groups were inoculated groupwise by the allantoic route with 5 ml of interferon per egg, 12, 6, and 3 hours before the virus inoculation, immediately after the virus inoculation and 3, 12 and 24 hours after the virus inoculation, respectively The residual 7 groups of eggs served as controls and were inoculated with control fluid at the above mentioned hours in relation to the virus inoculation

After the harvest the allantoic fluids were pooled groupwise and HA- and infectivity titrations were carried out immediately The results appear in Table 1 It can be seen, that the interferon had a marked in-

Of the 14 pools of allantoic fluids from interferon treated eggs in which a definite inhibition of virus synthesis occurred, the infectivity HA ratios could be calculated in five instances, i e in the fluids from the groups treated with 4 ml of undiluted interferon 12 and 24 hours after virus inoculation and from the groups which received 1 ml of undiluted interferon 3, 12 and 24 hours after virus inoculation Four of these fluids had higher infectivity HA ratios than the corresponding controls One of the fluids (1 ml of undiluted interferon given 3 hours after virus inoculation) had a ratio of 5 80 which is somewhat lower than in the corresponding control fluid (6 34) but well within the limits of the variation observed for all of the 28 control fluids Consequently all the 5 pools can be considered to consist predominantly of complete virus

The results obtained in the two experiments described above thus do not indicate that formation of incomplete virus can be induced by "passive administration of interferon

In the following experiment it was studied whether the in ovo production of incomplete virus would be affected when the endogenous formation of interferon was inhibited by hydrocortisone

This experiment was carried out under conditions *normally leading* to the formation of incomplete influenza virus, *i e by performing 4–7* serial passages with undiluted allantoic fluid virus (8) The experiment was *initiated* with two groups of each 10 embryos which had been pre-*incubated* for 10 days All eggs were inoculated with 0 5 ml portions of *freshly* harvested undiluted allantoic fluid virus containing 10[?] FID50 *influenza* B Lee per ml One hour after the virus inoculation one of the two groups (A) received 250 γ hydrocortisone while the other group (B) which *served* as control received physiological saline *After incubation* for 16 hours at 36° C the allantoic fluids were harvested and *pooled groupwise* From the two pools A and B thus obtained two parallel series each consisting of 6 successive passages [illegible] ited virus [illegible] ml of un- [illegible] 16 hours [illegible] γ hydrocortisone one [illegible] es were re inoculated [illegible] physiological sal[illegible] the freshly harve[illegible] HA titres of all p[illegible]

The result[illegible] it can be seen that the virus passages treated with hydrocortisone as well as the control passages yielded incomplete virus to almost the same extent The infectivity titres in the two series of passages are identical while the HA-titres in the series treated with steroid tend to be slightly lower than the HA titres of the corresponding controls Furthermore it can be seen that the sudden decline of haemagglutinating activity to submeasurable

vals, seven groups were inoculated with 1 ml interferon diluted 1:4 and seven groups with 1:16 diluted interferon For each group a control group which received non-interfering control fluid was included

All eggs received $10^{3.4}$ EID50 of influenza B Lee virus and were incubated at 36° C for 44 hours On harvest the fluids were pooled according to groups and titrated for infectivity and haemagglutinating activity The results are recorded in Table 2

As in the previous experiment a marked inhibition of the synthesis of virus was seen, when a dose of 4 ml undiluted interferon was administered either before or after the virus inoculation The inhibition of the synthesis of virus decreased rapidly with decreasing dose of interferon and no inhibition was observed in the eggs inoculated with 1 ml interferon diluted 1:4

TABLE 2

Virus Titres and Infectivity/HA Ratios in Allantoic Fluids from Eggs Having Received Varying Doses of Interferon at Varying Intervals of Time before and after the Inoculation of $10^{3.4}$ EID50 of Influenza B Lee Virus

Amount inoculated per egg	Inoculated with	Titres and ratios	Interferon inoculated at hours before and after the virus inoculation						
			−12	6	−3	0	+3	+12	+21
4 ml undiluted	Interferon	HA	<0.60*	<0.60	<0.60	<0.60	<0.60	<0.60	2.56
		EID50	5.4	5.4	5.8	6.4	5.6	7.0	8.4
		EID50/HA	>4.8	>4.8	>5.2	>5.8	>5.0	6.4	5.84
	Control	HA	2.58	3.18	2.66	3.11	2.66	3.86	3.71
		EID50	8.6	9.6	9.0	9.0	8.5	8.8	9.0
		EID50/HA	6.02	6.42	6.27	6.04	6.07	4.94	5.22
1 ml undiluted	Interferon	HA	<0.60	<0.60	<0.60	<0.60	1.20	1.73	3.16
		EID50	6.8	6.2	6.8	7.8	7.0	7.2	9.2
		EID50/HA	>6.2	>5.6	>6.2	>7.2	5.8	5.47	6.04
	Control	HA	2.88	3.18	2.66	3.11	2.66	3.86	3.71
		EID50	9.4	9.4	9.2	9.0	9.0	8.8	8.8
		EID50/HA	6.52	6.22	6.54	5.89	6.34	4.94	5.09
1 ml diluted 1:4	Interferon	HA	2.18	2.18	2.48	2.18	3.01	3.01	3.48
		EID50	8.3	8.5	7.7	8.1	8.0	8.7	9.1
		EID50/HA	6.12	6.32	5.22	5.92	4.99	5.69	5.62
	Control	HA	3.11	3.18	2.66	3.18	2.66	4.16	3.94
		EID50	8.3	8.5	8.7	8.9	8.5	8.8	9.1
		EID50/HA	5.19	5.32	6.04	5.72	5.87	4.94	5.16
1 ml diluted 1:16	Interferon	HA	3.09	3.38	3.24	2.94	3.01	2.94	3.24
		EID50	8.1	8.7	8.3	8.1	8.3	8.3	9.3
		EID50/HA	5.01	5.32	5.06	5.16	5.29	5.36	6.06
	Control	HA	2.81	2.88	2.66	3.03	3.11	3.78	3.78
		EID50	8.3	8.7	8.9	8.5	8.5	8.7	8.5
		EID50/HA	5.49	5.82	6.24	5.47	5.39	4.92	4.72

* All titres are recorded as $\log_{10}$

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All eggs received 10[3 4] EID50 of influenza B Lee virus and were incubated at 36° C for 44 hours On harvest the fluids were pooled according to groups and titrated for infectivity and haemagglutinating activity The results are recorded in Table 2

As in the previous experiment a marked inhibition of the synthesis of virus was seen, when a dose of 4 ml undiluted interferon was administered either before or after the virus inoculation The inhibition of the synthesis of virus decreased rapidly with decreasing dose of interferon and no inhibition was observed in the eggs inoculated with 1 ml interferon diluted 1 4

TABLE 2

Virus Titres and Infectivity/HA Ratios in Allantoic Fluids from Eggs Having Recieved Varying Doses of Interferon at Varying Intervals of Time before and after the Inoculation of 10[3 4] EID50 of Influenza B Lee Virus

Amount inoculated per egg	Inoculated with	Titres and ratios	Interferon inoculated at hours before and after the virus inoculation						
			−12	−6	−3	0	+3	+12	+24
4 ml undiluted	Interferon	HA	<0 60*	<0 60	<0 60	<0 60	<0 60	<0 60	2 56
		EID50	5 4	5 4	5 8	6 4	5 6	7 0	8 4
		EID50/HA	>4 8	>4 8	>5 2	>5 8	>5 0	6 4	5 84
	Control	HA	2 58	3 18	2 66	3 11	2 66	3 86	3 71
		EID50	8 6	9 6	9 0	9 0	8 5	8 8	9 0
		EID50/HA	6 02	6 42	6 27	6 04	6 07	4 94	5 22
1 ml undiluted	Interferon	HA	<0 60	<0 60	<0 60	<0 60	1 20	1 73	3 16
		EID50	6 8	6 2	6 8	7 8	7 0	7 2	9 2
		EID50/HA	>6 2	>5 6	>6 2	>7 2	5 8	5 47	6 04
	Control	HA	2 88	3 18	2 66	3 11	2 66	3 86	3 71
		EID50	9 4	9 4	9 2	9 0	9 0	8 8	8 8
		EID50/HA	6 52	6 22	6 54	5 89	6 34	4 94	5 09
1 ml diluted 1 4	Interferon	HA	2 18	2 18	2 48	2 18	3 01	3 01	3 48
		EID50	8 3	8 5	7 7	8 1	8 0	8 7	9 1
		EID50/HA	6 12	6 32	5 22	5 92	4 99	5 69	5 62
	Control	HA	3 11	3 18	2 66	3 18	2 66	4 16	3 94
		EID50	8 3	8 5	8 7	8 9	8 5	8 8	9 1
		EID50/HA	5 19	5 32	6 04	5 72	5 87	4 94	5 16
1 ml diluted 1 16	Interferon	HA	3 09	3 38	3 24	2 94	3 01	2 94	3 24
		EID50	8 1	8 7	8 3	8 1	8 3	8 3	9 3
		EID50/HA	5 01	5 32	5 06	5 16	5 29	5 36	6 06
	Control	HA	2 81	2 88	2 66	3 03	3 11	3 78	3 78
		EID50	8 3	8 7	8 9	8 5	8 5	8 7	8 5
		EID50/HA	5 49	5 82	6 24	5 47	5 39	4 92	4 72

* All titres are recorded as $\log_{10}$

is below that of the corresponding control pools Considering the inherent inaccuracy of interferon titrations (9) the differences in titres of interferon of the pools in passage No 6 cannot be considered significant As regard the remaining passages the interferon content is constantly lower in the fluids from the hydrocortisone treated eggs

The experiment thus shows that production of incomplete influenza virus proceeds as usual in embryonated eggs also when the production of interferon is significantly reduced by hydrocortisone

DISCUSSION

Based upon observations made in studies on the metabolic influence of interferon and different uncoupling agents *Isaacs et al* (4) suggested that the effect of interferon was due to an uncoupling of the nuclear oxydative phosphorylation thus interferon should act by inhibiting nuclear synthesizing mechanisms

Gresser (1) performed experiments with human amniotic cells and Sendai virus and demonstrated the production of interferon and of presumably incomplete Sendai virus He advanced the hypothesis that the formation of incomplete virus was due to a marked decrease of the synthesizing abilities of the nucleus while the synthesizing abilities of the cytoplasm was less affected and suggested that formation of incomplete influenza virus on serial passage of undiluted influenza virus might be due to reduced nuclear synthesizing abilities caused by interferon contained in the virus inoculum

In the present experiments addition of interferon to embryonated eggs inoculated with diluted influenza virus resulted in a marked inhibition of both the haemagglutinin and the infectivity titres of the allantoic fluids In many cases the synthesis of haemagglutinin was inhibited to such an extent that calculation of the infectivity HA ratio was impossible In cases with a moderate inhibition of the haemagglutinin synthesis an equally moderate inhibition of the infectivity was seen so that the infectivity HA ratio showed a value corresponding to complete virus

The experiment in which hydrocortisone was added to serial egg passages of undiluted influenza virus (Figs 1 and 2) showed that a dose of 250 γ di (17 hydrocorticosterone 21) phosphoricacidester sodium salt which in experiments with eggs inoculated with diluted influenza virus had a marked inhibiting effect upon the in ovo synthesis of interferon (9) also inhibited the synthesis of interferon when undiluted virus was passaged Thus in 6 out of 7 passages a distinctly decreased production of interferon was demonstrated in the hydrocortisone treated eggs In spite of this the production of incomplete influenza virus occurred in the usual manner

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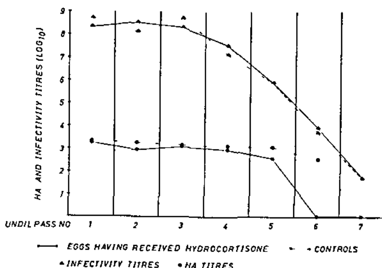


Fig 1

Influence of hydrocortisone upon the HA- and infectivity titres of allantoic fluids in a series of egg passages of undiluted influenza B Lee virus

values, which in the control series occurs in passage No 7, in the hydrocortisone treated series occurs already in passage No 6

Samples of each of the pools of allantoic fluids were dialysed for 48 hours to remove the hydrocortisone and subsequently they were heat-inactivated They were titrated for interferon after it had been established that the inactivated fluids were non-infectious and without haemagglutinating capacity As can be seen in Fig 2, the content of interferon in all pools of allantoic fluids from hydrocortisone treated eggs

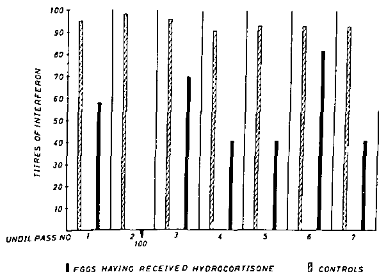


Fig 2

Influence of hydrocortisone upon the interferon titres of allantoic fluids in undiluted passages of influenza B Lee virus
The virus titres of the allantoic fluids are shown in Fig 1

is below that of the corresponding control pools. Considering the inherent inaccuracy of interferon titrations (9) the differences in titres of interferon of the pools in passage No 6 cannot be considered significant. As regard the remaining passages the interferon content is constantly lower in the fluids from the hydrocortisone treated eggs.

The experiment thus show that production of incomplete influenza virus proceeds as usual in embryonated eggs also when the production of interferon is significantly reduced by hydrocortisone.

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The experiment in which *hydrocortisone* was added to serial egg passages of undiluted influenza virus (Figs 1 and 2) showed that a dose of 250 γ di (17 hydrocorticosterone 21) *phosphoriacidester* sodium salt which in experiments with eggs inoculated with diluted influenza virus had a marked inhibiting effect upon the in ovo synthesis of interferon (9) also inhibited the *synthesis* of interferon when undiluted virus was passaged. Thus in 6 *out of* [illegible] passages a distinctly decreased production of interferon was demonstrated *in* the hydrocortisone treated eggs. In spite of this the production of *incomplete* influenza virus occurred in the usual manner.

Fig 1 shows that the content of haemagglutinin in pools of allantoic fluids from eggs treated with hydrocortisone showed a slight tendency

to be lower than in the corresponding control pools The differences are, however, hardly significant except for the 7th and possibly the 2nd passage This tendency towards *an inhibition of the production of haemagglutinin* has been described previously as occurring in egg passages of diluted influenza virus under the influence of steroids (5, 9) The fact that the content of haemagglutinin declines to submeasurable values in passage No 6 in the hydrocortisone treated eggs, while the decline does not occur until passage No 7 in the controls may possibly also be attributed to steroid inhibition of the production of haemagglutinin It may be accidental, however, since such variations somtimes occur in otherwise identical series of passages

SUMMARY

Experiments were undertaken to elucidate and possibly substantiate the hypothesis of interferon being an integral intermediary in the formation of incomplete influenza virus by investigating whether addition of interferon to in ovo passages of diluted influenza B Lee virus would result in a production of incomplete virus A decline was found in haemagglutinin- as well as in infectivity titres, but the virus yield was found to be fully infectious, *i e* complete

It was furthermore studied whether hydrocortisone inhibition of the production of interferon in serial in ovo passages of undiluted influenza B Lee virus resulted in a decrease of the degree of incompleteness of the virus In spite of distinctly decreased production of interferon, the degree of incompleteness of the virus yield was not changed

The present experiments are thus not compatible with the hypothesis that interferon plays an integral rôle in the formation of incomplete virus

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STUDIES ON THE ANTIGENIC STRUCTURE OF THE 80/81 COMPLEX OF STAPHYLOCOCCUS AUREUS

3. *Purification and Chemical Characterization of a Major Polysaccharide Precipitinogen*

By

TOR HOFSTAD

Received 2.viii.64

In a previous paper the agglutinogens of *Staphylococcus aureus* strains within the 80/81 complex were reported (30). The phage type 80/81/82 KS6 strain 263, widely used in that study, has also been examined for precipitinogens by double diffusion in agar against homologous and heterologous immune sera. These investigations revealed a previously unrecognized agar precipitation line. In preliminary studies the corresponding antigen was found to be chemically very similar to although serologically different from polysaccharide A (21). The present paper deals with the purification and chemical characterization of this antigen, provisionally named polysaccharide 263. The antigenic properties of the purified antigen will be reported in a forthcoming paper (32).

Polysaccharide A was purified chromatographically by *Haukenes* (21, 22) in our laboratory from extracts of crushed microbes. On chemical analyses the purified material was found to be a complex of ribitol teichoic acid and mucopeptide (23, 24, 25). Identical serological reactivity was demonstrated when polysaccharide A and ribitol teichoic acid from cell walls of *Staph. aureus* strain H were compared against *Staph. aureus* immune serum (26, 27).

The teichoic acids are polymers of glycerol or ribitol phosphate containing glycosidically linked sugar and esterified D-alanine residues (2) found in Gram positive bacteria, primarily in cell walls. The wall teichoic acid in *Staph. aureus* is a ribitol teichoic acid containing alpha or beta linked N-acetylglucosamine residues, or both (3, 48).

MATERIALS

Strains 263 and 263 KS6 6 (the latter obtained by *in vitro* lysogenization of strain 263) have been described earlier (30, 31). The bacteria were grown for 18 hrs on

to be lower than in the corresponding control pools The differences are, however, hardly significant except for the 7th and possibly the 2nd passage This tendency towards an inhibition of the production of hae magglutinin has been described previously as occurring in egg passages of diluted influenza virus under the influence of steroids (5, 9) The fact that the content of haemagglutinin declines to submeasurable values in passage No 6 in the hydrocortisone treated eggs, while the decline does not occur until passage No 7 in the controls may possibly also be attributed to steroid inhibition of the production of haemag glutinin It may be accidental, however, since such variations somtimes occur in otherwise identical series of passages

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MATERIALS

Strains 263 and 263 KS6-6 the latter obtained by *in vitro* lysogenization of strain 263 have been described earlier (30 31) The bacteria were grown for 18 hrs on

nutrient agar plates and harvested by scraping The harvested organisms were either used immediately or after storage at —25° C

Purified polysaccharide A ribitol teichoic acid from cell walls of *Staph aureus* strain 3528 (41) (furnished by Professor *J L Strominger* St Louis) and glycerol teichoic acid from cell walls of *Staph albus* NCTC 7944 (8) (furnished by Professor *J Baddiley* Newcastle) have been used as reference substances The ribitol teichoic acid contained N acetylglucosamine while the sugar in the glycerol teichoic acid was N acetylgalactosamine

METHODS

Agar Precipitation

The agar medium and the plates were prepared as described by *Haukenes & Oeding* (29) The circular basins used had a diameter of 6 mm and were 5 mm apart Unless otherwise stated the plates were incubated at 4° C for four to five days and read daily Sometimes when it was suspected that strong antigens diffused towards weak sera the first reading was made after four to six hours incubation This procedure which was based on preliminary experiments with varying size and arrangement of the basins and varying incubation temperature gave fairly rapid evolvement of distinct well separated bands of precipitation and was in addition economic

The *immune sera* used were produced by intravenous injections of formalin killed organisms (42)

Fractionation Methods

Preparation of crude polysaccharide The optimal conditions for extraction of antigenic materials were examined in preliminary experiments Different buffers were tried for extraction of intact and crushed (Hughes bacteria press (34)) microbes and the extraction process was studied for several days at 4 20 and 37° C The procedure finally adopted was prolonged extraction of intact bacteria at 37° C with 0 2 M acetate buffer of ionic strength 0 3 and pH 5 8

The further preparation of the crude polysaccharide was as described by *Haukenes et al* (28) Dilute hydrochloric acid was added to the extract and the precipitate formed at pH 4 2 was removed The crude polysaccharide material was precipitated with five volumes of ethanol at pH 5 2 This procedure was repeated until no more precipitate was formed at pH 4 2

Ion exchange columns Diethylaminoethyl (DEAE) cellulose (Eastman) 100–230 *mesh particle size was prepared as described by Peterson & Sober (45) The pro*cedure for running the column was principally that of *Haukenes* (21) Gradient elution was carried out with NaCl and the system was arranged to give a continuous increase of the gradient from zero to 1 M

Dowex 1 (Fluka) 8 per cent cross linkage 200–400 mesh particle size and Amber [illegible] ge 100–230 mesh particle size were prepared

[illegible] harmacia) were used for gel filtration The directions from the manufacturers [illegible] tubes (Kalle A/G)

Paper electrophoresis was carried out in N acetic acid in acetate buffer of ionic strength 0 3 and pH 5 8 and in veronal buffer of ionic strength 0 1 and pH 8 6 The apparatus used was modified from that of *Foster* (10)

The procedures for application running and elution of the material under investigation were as described in (26) but the eluates were examined by agar precipitation instead *of the ring test*

Immunoelectrophoresis was performed with LKB 6800 A Immunoelectrophoretic Equipment according to the description in the Operating Manual

Paper Chromatography

Hydrolysis was performed in sealed tubes in

1 0 1 N HCl for 2 hrs at 100° C
2 3 N HCl for 3 hrs at 100° C
3 6 N HCl for 16 hrs at 105° C
4 N NH_4OH for 5 mins at 100 C

The dried materials were made up in distilled water and subjected to chromato graphic analysis Circular chromatography with Whatman No 1 filter paper as the stationary phase was carried out by a method of *Giri & Rao* (16) as modified by *Grov & Musaker* (17)

Developing Solvents

A BuOH HAc H_2O (4 1 1) (46)
B PhOH H_2O (4 1 w v) (6)
C PrOH NH_3 (d 0 88) H_2O (6 3 1) (20)
D Iso-PrOH 2 N HCl (65 35) (50)

Spray Reagents

a Ninhydrin 0 4 per cent in acetone containing 2 per cent acetic acid for the detection of amino acids and amino sugars (39)
b Na periodate benzidine for sugar alcohols (5)
c The Elson Morgan reagents modified by *Partridge* (43) for amino sugars
d Aniline hydrogen phthalate in water saturated butanol for detection of aldo hexoses and aldopentoses (44)
e Isatine for detection of proline and hydroxyproline (1)
Oxidation of amino sugars with ninhydrin was carried out as described by *Stoffyn & Jeanloz* (49)

Analytical Methods

Nitrogen was determined by the micro Kjeldahl method as descr bed in (?)

[illegible]

... Hagedorn-Jensen method as modified by *Hanes* (19)

[illegible]

an ...
ca ...

as described by *Giri et al* (1 ...
Th ...

[illegible]

All measurements of optic ...
photometer with 1 0 cm cells

EXPERIMENTAL PROCEDURE AND RESULTS

A *Precipitinogens Found in Strain 263*

Suspensions and extracts of live and crushed 263 microbes were examined by double diffusion in agar against ten homologous immune sera Three principal lines were found (*Fig 1*) Line A is the poly saccharide A line as this line showed complete fusion with the line produced by purified polysaccharide A Line B is the specific line produced by polysaccharide 263 hereafter referred to as the polysaccharide 263 line These lines appeared after 12 to 18 hrs incubation at 4° C In cubation at 37° C hastened the appearence of the lines but after in cubation overnight at 37° C they were both less distinct and tended to disappear into the serum basin The third line line C, appeared in most

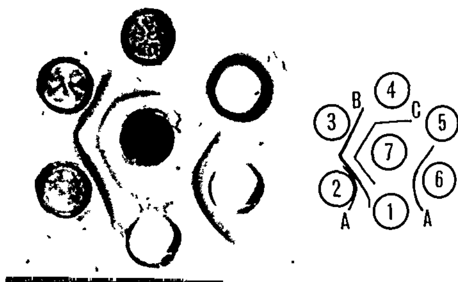


Fig 1

The principal lines produced by *Staph aureus* strain 263 on agar gel diffusion 263 microbes and homologous immune sera in wells 7, 2 and 3 respectively Serum Cowan I, pooled human serum, serum Wood 46 and purified polysaccharide A in wells 4, 5, 6 and 1 respectively

A – polysaccharide A line   B – polysaccharide 263 line

experiments after two days incubation, and was fully developed by the third day This line, which was best evolved using crushed microbes and incubation at 37° C, corresponds to a widely distributed antigen as the line seems to be produced by all the coagulase positive staphylococci so far examined

A weak protein A (antigen A) line (18) was obtained with some 263 sera No lines were demonstrated when suspensions or extracts of live 263 microbes were examined against rabbit sera before immunization with strain 263

Polysaccharide 263 proved to be the major precipitinogen of strain 263 When, for instance, 1 g wet weight of crushed 263 microbes was extracted overnight with 5 ml acetate buffer, a distinct polysaccharide 263 line appeared in dilutions of the extract up to 1 250 The polysaccharide A line was given by the same extract diluted 1 10, and line C by undiluted extract only

B *Purification of Polysaccharide 263*

Preliminary Experiments

The first attempts to purify polysaccharide 263 were made using crude polysaccharide from strain 263

In a representative experiment the crude material was prepared as described (cf methods) and subjected to gradient elution on DEAE

cellulose columns The fractions were treated as described below The de salted and freeze dried polysaccharide dissolved in saline, produced a distinct polysaccharide A line in addition to the stronger polysac charide 263 line

Several experiments were carried out in order to separate the two polysaccharide antigens They were all without success Polysaccharide 263 was eluted from Dowex 1 columns within the same range of effluent molarity as polysaccharide A using gradient elution with ammonium formate Both polysaccharides went straight through an Amberlite IR 120 column When the purified polysaccharide material dissolved in a minimum volume of distilled water, was applied to columns of Sephadex G 50 Medium and Sephadex G 50 Fine and collected in 1 ml fractions the peak concentrations of the two polysaccharide antigens were found in the same fractions

The purified polysaccharide material was also subjected to immuno electrophoresis at alkaline and acid pH The relative positions of the polysaccharide 263 and polysaccharide A lines were the same as by ordinary agar precipitation indicating that the two polysaccharide anti gens had not been separated electrophoretically

In some of the preliminary experiments the eluates from DEAE cellulose columns had to be de-salted more than once on Sephadex G 50 or Sephadex G 25 columns De salting by dialysis against running water was therefore used in further experiments

No chemical analyses were made on the purified polysaccharide material from strain 263

Purification of Polysaccharide 263 from Strain 263 KS6 6

The inability to separate polysaccharide 263 from polysaccharide A directed further investigations on the purification of polysaccharide 263 towards *Staph aureus* strains producing no polysaccharide A line Strain 263 KS6 6 was found to be ideal being a phage variant of strain 263 with the same antigenic apparatus except for the polysaccharide A line (31) which was demonstrated with concentrated extracts only or not at all

In the present experiment 36 g wet weight 263 KS6–6 microbes were extracted for 7 days in 360 ml acetate buffer at 37° C The yield of lyo philized crude polysaccharide was 725 mg The polysaccharide 263 line was obtained in dilutions of the lyophilized crude polysaccharide up to 1 2 5 × 10 while line C (cf Figure 1) could not be demonstrated in dilutions higher than 1 10 A 1 per cent solution produced no poly saccharide A line with immune serum Wood 46 When the 1 per cent solution was examined against immune sera to our 13 type strains (30) another weak band (designated line D on Fig 2) appeared with a few immune sera (immune sera 3647 and 1503) midway between the basins close to line C



Fig 1

The principal lines produced by *Staph aureus* strain 263 on agar gel diffusion 263 microbes and homologous immune sera in wells 7, 2 and 3 respectively Serum Cowan I, pooled human serum, serum Wood 46 and purified polysaccharide A in wells 4, 5, 6 and 1 respectively

A – polysaccharide A line B polysaccharide 263 line

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A weak protein A (antigen A) line (18) was obtained with some 263 sera No lines were demonstrated when suspensions or extracts of live 263 microbes were examined against rabbit sera before immunization with strain 263

Polysaccharide 263 proved to be the major precipitinogen of strain 263 When, for instance, 1 g wet weight of crushed 263 microbes was extracted overnight with 5 ml acetate buffer, a distinct polysaccharide 263 line appeared in dilutions of the extract up to 1 250 The polysaccharide A line was given by the same extract diluted 1 10, and line C by undiluted extract only

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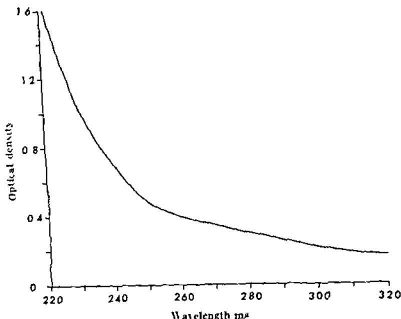


Fig 3
Ultraviolet absorption spectrum of purified polysaccharide 263 in 0 5 per cent aqueous solution

The highest dilution of purified polysaccharide 263 producing the specific line of precipitation was 1: 10[6] No other lines were found in concentrations up to 5 mg per ml

C *Chemical Characterization of Purified Polysaccharide 263*

The purified polysaccharide was a white, hygroscopic powder, which was readily dissolved in water giving a clear, colourless solution The pH of a 1 per cent aqueous solution was 4 8 when the pH of the water was 5 1

The polysaccharide did not pass the cellophane membrane on dialysis, and went straight through columns of Sephadex G-25 and Sephadex G-50 Some overlapping with salt containing fractions was sometimes observed

Biuret, Dische's and Bial's tests, carried out on a 0 1 per cent solution of the polysaccharide, were all negative The same solution showed a very weak Molisch test

Specific optical rotation was measured with tubes of 20 cm length at 21° C An aqueous solution of polysaccharide 263 showed a rotation of +60 0°

The ultraviolet absorption spectrum of a 0 5 per cent solution of the

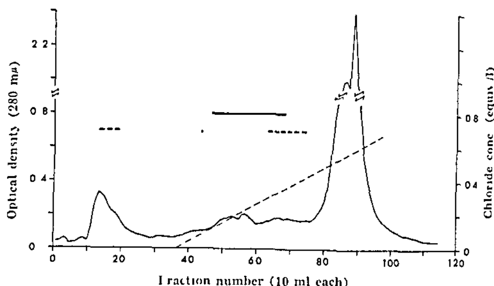


Fig 2

Chromatography of crude polysaccharide from strain 263 KS6 6 on DEAE cellulose

———— Polysaccharide 263
— — — — Antigenic material corresponding to line C
Antigenic material corresponding to line D
— — — — Chloride concentration

The crude polysaccharide, dissolved in 0 02 M phosphate buffer of pH 7 4, was applied to a 2 5 × 20 cm column of DEAE cellulose The resin had previously been equilibrated with the same buffer The effluent fractions, 10 ml each, were examined by agar precipitation against 263 serum and a 3647 serum giving line D Polysaccharide 263 was eluted between molarities of sodium chloride of 0 13 and 0 38 (Fig 2) The fractions producing the polysaccharide 263 line but not the weak line D, *i e* between molarities of sodium chloride of 0 15 and 0 38, were combined, and the volume reduced by distillation under reduced pressure The polysaccharide material was precipitated with ethanol, dissolved in phosphate buffer and applied to another DEAE cellulose column The conditions for running this column were arranged to give a slower increase in the molarity of sodium chloride Polysaccharide 263 was eluted within the same range of effluent molarity as before Antigenic material corresponding to line C was eluted in the region of 0 3 to 0 4 M sodium chloride, and showed less overlapping with polysaccharide 263 than during the first run The fractions producing the polysaccharide 263 line only, *i e* the positive fractions up to a salt concentration just below 0 3 M sodium chloride, were combined and subjected to a third column of DEAE cellulose All positive fractions eluted from this column were combined and de-salted by dialysis against running water for 48 hrs The yield of lyophilized purified polysaccharide 263 was 54 mg

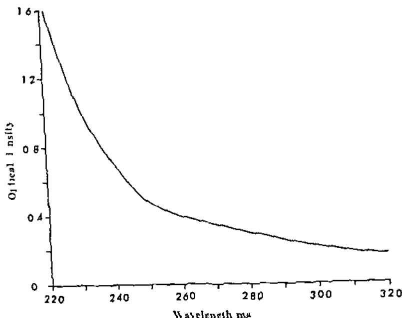


Fig 3
Ultraviolet absorption spectrum of purified polysaccharide 263 in 0 5 per cent aqueous solution

The highest dilution of purified polysaccharide 263 producing the specific line of precipitation was 1 10^6 No other lines were found in concentrations up to 5 mg per ml

C *Chemical Characterization of Purified Polysaccharide 263*

The purified polysaccharide was a white hygroscopic powder which was readily dissolved in water giving a clear colourless solution The pH of a 1 per cent aqueous solution was 4 8 when the pH of the water was 5 1

The polysaccharide did not pass the cellophane membrane on dialysis and went straight through columns of Sephadex G 25 and Sephadex G 50 Some overlapping with salt containing fractions was sometimes observed

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The ultraviolet absorption spectrum of a 0 5 per cent solution of the

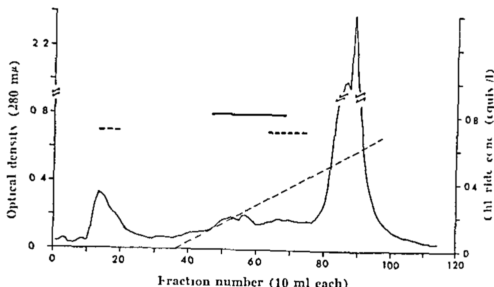


Fig. 2

Chromatography of crude polysaccharide from strain 263-KS6-6 on DEAE cellulose.

——————— Polysaccharide 263
– – – – – – Antigenic material corresponding to line C
.... Antigenic material corresponding to line D
– – – – – – Chloride concentration

The crude polysaccharide, dissolved in 0.02 M phosphate buffer of pH 7.4, was applied to a 2.5 × 20 cm column of DEAE cellulose. The resin had previously been equilibrated with the same buffer. The effluent fractions, 10 ml each, were examined by agar precipitation against 263 serum and a 3647 serum giving line D. Polysaccharide 263 was eluted between molarities of sodium chloride of 0.13 and 0.38 (Fig. 2). The fractions producing the polysaccharide 263 line but not the weak line D, *i.e.* between molarities of sodium chloride of 0.15 and 0.38, were combined, and the volume reduced by distillation under reduced pressure. The polysaccharide material was precipitated with ethanol, dissolved in phosphate buffer and applied to another DEAE cellulose column. The conditions for running this column were arranged to give a slower increase in the molarity of sodium chloride. Polysaccharide 263 was eluted within the same range of effluent molarity as before. Antigenic material corresponding to line C was eluted in the region of 0.3 to 0.4 M sodium chloride, and showed less overlapping with polysaccharide 263 than during the first run. The fractions producing the polysaccharide 263 line only, *i.e.* the positive fractions up to a salt concentration just below 0.3 M sodium chloride, were combined and subjected to a third column of DEAE cellulose. All positive fractions eluted from this column were combined and de-salted by dialysis against running water for 48 hrs. The yield of lyophilized purified polysaccharide 263 was 54 mg.

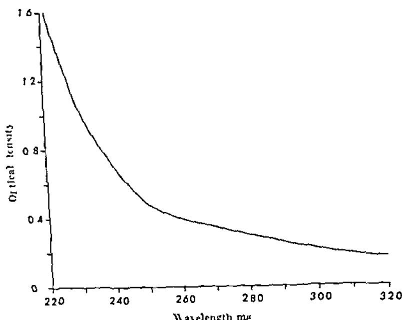


Fig 3
Ultraviolet absorption spectrum of purified polysaccharide 263 in 0 5 per cent aqueous solution

The highest dilution of purified polysaccharide 263 producing the specific line of precipitation was 1 10^6 No other lines were found in concentrations up to 5 mg per ml

C *Chemical Characterization of Purified Polysaccharide 263*

The purified polysaccharide was a white, hygroscopic powder, which was readily dissolved in water giving a clear, colourless solution The pH of a 1 per cent aqueous solution was 4 8 when the pH of the water was 5 1

The polysaccharide did not pass the cellophane membrane on dialysis, and went straight through columns of Sephadex G 25 and Sephadex G 50 Some overlapping with salt containing fractions was sometimes observed

Biuret Dische s and Bial s tests, carried out on a 0 1 per cent solution of the polysaccharide, were all negative The same solution showed a very weak Molisch test

Specific optical rotation was measured with tubes of 20 cm length at 21° C An aquous solution of polysaccharide 263 showed a rotation of + 60 0°

The ultraviolet absorption spectrum of a 0 5 per cent solution of the

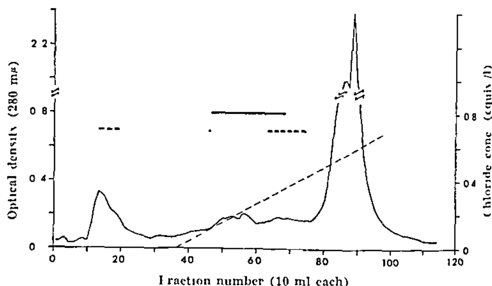


Fig 2

Chromatography of crude polysaccharide from strain 263 KS6 6 on DEAE cellulose

——————— Polysaccharide 263
— — — — — Antigenic material corresponding to line C
Antigenic material corresponding to line D
— — — — — Chloride concentration

The crude polysaccharide, dissolved in 0 02 M phosphate buffer of pH 7 4, was applied to a 2 5 × 20 cm column of DEAE cellulose The resin had previously been equilibrated with the same buffer The effluent fractions, 10 ml each, were examined by agar precipitation against 263 serum and a 3647 serum giving line D Polysaccharide 263 was eluted between molarities of sodium chloride of 0 13 and 0 38 (Fig 2) The fractions producing the polysaccharide 263 line but not the weak line D, *i e* between molarities of sodium chloride of 0 15 and 0 38, were combined, and the volume reduced by distillation under reduced pressure The polysaccharide material was precipitated with ethanol, dissolved in phosphate buffer and applied to another DEAE cellulose column The conditions for running this column were arranged to give a slower increase in the molarity of sodium chloride Polysaccharide 263 was eluted within the same range of effluent molarity as before Antigenic material corresponding to line C was eluted in the region of 0 3 to 0 4 M sodium chloride, and showed less overlapping with polysaccharide 263 than during the first run The fractions producing the polysaccharide 263 line only, *i e* the positive fractions up to a salt concentration just below 0 3 M sodium chloride, were combined and subjected to a third column of DEAE cellulose All positive fractions eluted from this column were combined and de salted by dialysis against running water for 48 hrs The yield of lyophilized purified polysaccharide 263 was 54 mg

the paper for the quantitative determination of amino acids Owing to this, the glucosamine spot became large and sometimes showed a slight overlapping with the lysine spot Hence, somewhat varying results were obtained in different experiments, but in no instances was the amino acid content of the purified polysaccharide found to exceed 3 5 per cent The results obtained in a representative determination are listed in Table 1

TABLE 1

Quantitative Analysis of Purified Polysaccharide 263

	Per cent
Nitrogen	3 14
Phosphorus	6 12
Reducing sugars (as glucose)	24 30
Hexosamines (as glucosamine)	18 50
Glycine	1 3
α alanine	0 7
Lysine	0 8
Glutamic acid	0 5

Paper Electrophoresis

The examinations so far indicated that polysaccharide 263 was composed of ribitol teichoic acid and peptide Serological studies on the purified product strongly indicated that the chemical basis of the precipitating activity was the teichoic acid moiety (32) Polysaccharide 263 was therefore subjected to paper electrophoresis in order to separate the peptide from the serologically active teichoic acid moiety

Polysaccharide 263 migrated towards the anode both at pH values of 2 35 5 8 and 8 6 In N acetic acid, pH 2 35, the substance migrated 12 to 18 cm in 3 hrs The distance of migration after 1½ hrs in the acetate and veronal buffers was 7 to 16 and 5 to 10 cm resptively The potential was the same, 500 V, i e 17 V/cm, in all experiments

The serologically active material was eluted with water, hydrolysed in 6 N HCl for 16 hrs at 105° C, and subjected to paper chromatography in solvent system A Alanine, glycine lysine [illegible] cosa- [illegible] that [illegible] f the [illegible] had not been achieved

DISCUSSION

Strain 263 KS6–6 was found to be well suited for the preparation of polysaccharide 263 The antigen was easily extracted from whole microbes and the purified product was not contaminated with other precipitinogens

The fractionation on DEAE cellulose columns was accompanied by loss of ultraviolet light absorbing material and increasing serological activity The recovery of only 54 mg of purified polysaccharide (titre 1 10^{6}) from 725 mg of crude extract (titre 1 2 · × 10) showed, how-

purified polysaccharide is shown in Fig 3 No peaks were found between 225 and 320 mμ The extinction at 260 and 280 mμ was 0 408 and 0 296, respectively

The agar precipitation titre against immune serum 263 remained unchanged after tryptic digestion (Trypure Novo) of the polysaccharide and autoclaving for 2 hrs at 120° C

Paper Chromatography

A 1 per cent solution of the 0 1 N hydrochloric acid hydrolysate of purified polysaccharide 263 was examined chromatographically for purines and pyrimidines in the developing system D, using a 0 1 per cent solution of a hydrolyzed sample of nucleic acids (from Thymus Gland, BDH) as control While the nucleic acid preparation gave strong adenine and guanine spots when examined under an ultraviolet lamp (2015 Chromatolite 2537 A, Shandon), nothing was observed in the polysaccharide 263 sample

3 N hydrochloric acid hydrolysates were examined for sugar alcohols and free sugars in system C, and for amino sugars in systems A and C Hydrolysed samples of ribitol and glycerol teichoic acids were used as references Ribitol and anhydroribitol were detected with sodium-periodate-benzidine, and glucosamine (or galactosamine) with the modified Elson-Morgan reagent Muramic acid was not demonstrated in samples of up to 0 6 mg hydrolysed polysaccharide 263 When the acid hydrolysate was treated with ninhydrin, arabinose (identified in system C), was formed, showing that the amino sugar was glucosamine Free sugars were not found in aniline hydrogen phthalate-treated chromatograms

Alanine, glycine, lysine and glutamic acid (and glucosamine) were identified when 6 N hydrochloric acid hydrolysates were run in systems A and B

The alkaline hydrolysate of polysaccharide 263 was examined chromatographically in system C No spots were detected in ninhydrin-treated chromatograms An alkaline hydrolysate of ribitol teichoic acid produced a strong alanine spot

On the chromatograms studied, no spots were observed which could not be identified

Quantitative Analyses

The results from the quantitative analyses have been compiled in Table 1

When measuring the hexosamine quantity, the extinction was also recorded at 505 mμ immediately after finishing the experimental procedure and after 24 hrs A slight increase (10 per cent) was found after 24 hrs, indicating a trace of muramic acid

Up to 0 5 mg of hydrolysed polysaccharide 263 had to be applied to

the paper for the quantitative determination of amino acids Owing to this, the glucosamine spot became large and sometimes showed a slight overlapping with the lysine spot Hence, somewhat varying results were obtained in different experiments, but in no instances was the amino acid content of the purified polysaccharide found to exceed 3 5 per cent The results obtained in a representative determination are listed in Table 1

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DISCUSSION

Strain 263 KS6 6 was found to be well suited for the preparation of polysaccharide 263 The antigen was easily extracted from whole microbes and the purified product was not contaminated with other precipitinogens

The fractionation on DEAE cellulose columns was accompanied by loss of ultraviolet light absorbing material and increasing serological activity The recovery of only 54 mg of purified polysaccharide (titre 1 10) from 725 mg of crude extract (titre 1 2 5 × 10) showed, how-

purified polysaccharide is shown in Fig 3 No peaks were found between 225 and 320 mμ The extinction at 260 and 280 mμ was 0 408 and 0 296, respectively

The agar precipitation titre against immune serum 263 remained unchanged after tryptic digestion (Trypure Novo) of the polysaccharide and autoclaving for 2 hrs at 120° C

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equimolar amounts of glucosamine and phosphate (3 13 48) The low hexosamine value found in the present investigation may be explained by errors in acid hydrolysis (2 11) This is compatible with the nitrogen content of the purified material which cannot be accounted for by the figures obtained for hexosamines and amino acids

It is reasonable to believe that polysaccharide 263 was released enzymatically from intact bacteria during the period of extraction *Haukenes* finding of minimal liberation of polysaccharide 263 from heated cells (26) was found to be true also for polysaccharide 263 It is worth noting that an acetate buffer of the same ionic strength and pH as in the present study was used by *Mitchell & Moyle* (40) for release of protoplasts from *Staph aureus* The nature of the lytic enzyme (or enzymes) responsible for the release of polysaccharide 263 is obscure The great similarity of polysaccharide 263 to the teichoic acid glycopeptide of *Ghuysen & Strominger* points however to a staphylococcal acetylmuramidase (14)

Polysaccharide 263 displayed one striking difference from polysaccharide A i e being dextrorotatory This made the author think that the serological difference between the two antigens depended on the nature of the glucosamine linkage in the ribitol teichoic acid moiety This assumption has recently been confirmed (32)

SUMMARY

A major precipitinogen of *Staphylococcus aureus* strain 263 (phage type 80 81 82 KS6) has been isolated from extracts of intact bacteria of the variant strain 263 KS6 6 The antigen was precipitated with ethanol after removal of nucleoproteins and purified by gradient elution on diethylaminoethyl (DEAE) cellulose columns The purified material produced a specific agar precipitation line with homologous immune serum in dilutions up to 1 10⁵

By qualitative and quantitative chemical analyses the purified product was found to be a polysaccharide made up of alanine free ribitol teichoic acid firmly linked to a small peptide fraction The sugar of the teichoic acid moiety was glucosamine and the peptide was composed of alanine glycine lysine and glutamic acid

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ever, that considerable amounts of the serologically active principle were lost during the process of purification The positive fractions which were discarded because of contamination with other antigens, cannot alone account for the poor recovery

The negative tests on purines and pyrimidines and the negative Bial s and Dische's tests exclude the presence of nucleic acid derivatives in the purified product This is in accordance with the absorption spectrum shown in Fig 3

The demonstration of glucosamine, ribitol and anhydroribitol, and of alanine, glycine, lysine and glutamic acid, together with the high content of organic phosphorus, shows that polysaccharide 263, like polysaccharide A (24) is composed of glucosaminylribitol teichoic acid, and a peptide The close similarity to polysaccharide A was clearly demonstrated by the failure to separate the two polysaccharide antigens chemically Analogous to polysaccharide A (26) the purified polysaccharide 263 was found to be completely free of alkali-labile alanine

Muramic acid was not demonstrated by paper chromatography, but the slight increase in the extinction at 505 mμ may indicate that minute amounts of this amino sugar were present in the purified product A slight increase in the absorbancy at the same wave length was, however, observed by *Haukenes* (23), on examining the reliability of the method of *Rondle & Morgan*, with a standard sample of 25 μ glucosamine

The unsuccessful attempts to separate the peptide from the teichoic acid moiety in polysaccharide 263 by electrophoresis, indicate that the two structural components are firmly linked to each other Analogous findings have been made for polysaccharide A (26) and for the staphylococcal teichoic acid-glycopeptide complex described by *Ghuysen & Strominger* (13) Similar complexes have also been demonstrated in cell walls of group A and C streptococci (37, 38), groups B and C of *L casei* (36), and in cell walls of *B megaterium* (12)

The results obtained by quantitative analyses of polysaccharide 263 indicate that less than three quarters of the purified material has been accounted for The most likely explanation of the incomplete recovery is that one or more data have not been quantitative The gross quantitative composition of the teichoic acid-peptide complex has, nevertheless, been established by the analyses carried out The quantities found, expressed as moles per mole of phosphate hexosamine (i e glucosamine) 0 57, glycine 0 087, alanine 0 040, lysine 0 027 and glutamic acid 0 016, show that the main constituent of polysaccharide 263 is alanine-free teichoic acid The small peptide moiety is most likely derived from the cell wall About the same molar ratios have been found for the same four amino acids in preliminary experiments on isolated cell walls from strain 263 (33)

The repeating unit in acid extracted or enzymatically released ribitol teichoic acid polymers from *Staph aureus* has been found to contain

equimolar amounts of glucosamine and phosphate (3 13 48) The low hexosamine value found in the present investigation may be explained by errors in acid hydrolysis (2 11) This is compatible with the nitrogen content of the purified material which cannot be accounted for by the figures obtained for hexosamines and amino acids

It is reasonable to believe that polysaccharide 263 was released enzymatically from intact bacteria during the period of extraction *Haukenes* finding of minimal liberation of polysaccharide 263 from heated cells (26) was found to be true also for polysaccharide 263 It is worth noting that an acetate buffer of the same ionic strength and *p*H as in the present study was used by *Mitchell & Moyle* (40) for release of protoplasts from *Staph aureus* The nature of the lytic enzyme (or enzymes) responsible for the release of polysaccharide 263 is obscure The great similarity of polysaccharide 263 to the teichoic acid glycopeptide of Ghuysen & *Strominger* points however to a staphylococcal acetylmuramidase (14)

Polysaccharide 263 displayed one striking difference from polysaccharide A i e being dextrorotatory This made the author think that the serological difference between the two antigens depended on the nature of the glucosamine linkage in the ribitol teichoic acid moiety This assumption has recently been confirmed (32)

SUMMARY

A major precipitinogen of *Staphylococcus aureus* strain 263 (phage type 80 81 82 KS6) has been isolated from extracts of intact bacteria of the variant strain 263 KS6 6 The antigen was precipitated with ethanol after removal of nucleoproteins and purified by gradient elution on *diethylaminoethyl (DEAE)* cellulose columns The purified material produced a specific agar precipitation line with homologous immune serum in dilutions up to 1 10

By qualitative and quantitative chemical analyses the purified product was found to be a polysaccharide made up of alanine-free ribitol teichoic acid firmly linked to a small peptide fraction The sugar of the teichoic acid moiety was glucosamine and the peptide was composed of alanine glycine lysine and glutamic acid

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Fig 1

Section of a mouse kidney rudiment after 9 days infection with polyoma virus *in vitro* A part of a secretory tubule (T) is seen without signs of viral infection The surrounding mesenchyme shows advanced viral lesions with disintegrating cells, lysosomes (L) and numerous viral particles (V) in nuclei and cytoplasm
Magnification × 6000

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VIRAL SUSCEPTIBILITY AND EMBRYONIC DIFFERENTIATION

5 *Localization of Virus-Like Particles in Mouse Kidney Rudiment Infected with Polyoma Virus*

By

JORMA WARTIOVAARA, LAURI SAXEN and TAPANI VAINIO[1]

Received 16 vii 64

A possible model for studying the restriction of viral susceptibility during embryogenesis has been suggested by our earlier investigations on developing mouse kidney The progenitors of epithelial tubulus cells seem to be highly susceptible to polyoma virus whereas maturing cells resist infection with this agent (*Saxen et al* 1962, *Vainio et al* 1963a b) These conclusions were drawn from observations made by employing light microscopy and immunofluorescence only, hence, it seemed important to confirm the results by using the electron microscope to determine the tissue localization of the virus

METHODS

Mice 13 day-old embryos of random bred Swiss mouse were used The O day was evidenced by vaginal plugging

Organ cultures The kidneys were removed from the embryos and placed on a Millipore filter on a Trowell type metal screen The culture medium was Eagle's basal medium in Earle's balanced salt solution with 10 per cent inactivated calf serum and 0 072 M of sodium bicarbonate added The cultures were incubated in small petri dishes in 5 per cent CO_2 in air (*Saxen et al* 1962)

Infection of the cultures Organ rudiments were adsorbed with a 1 10 dilution of the original stock virus, SE polyoma The stock virus, prepared in mouse fibro blast cultures, had an HA titre of 1 1024 5 cc of viral dilution was used The culture medium was not renewed during the experiments

Electron microscopy The kidney rudiments were fixed for 30 minutes in cold 1 per cent OsO_4 buffered to pH 7 3 with veronal acetate (*Palade* 1952) Dehydration was carried out in a graded series of increasing concentrations of ethyl alcohol and the tissues were embedded in Epon according to *Luft* (1961) Thin sections were cut [illegible] were picked up [illegible] l citrate em plo[illegible] Elmiskop I electron microscope Electron micrographs were made at original magnifications of 1900 to 80000 diameters and enlarged as desired

Experimental series For the study of viral lesions in kidney rudiments 13 day-old foetal mouse kidneys were infected with polyoma virus Cultured rudiments

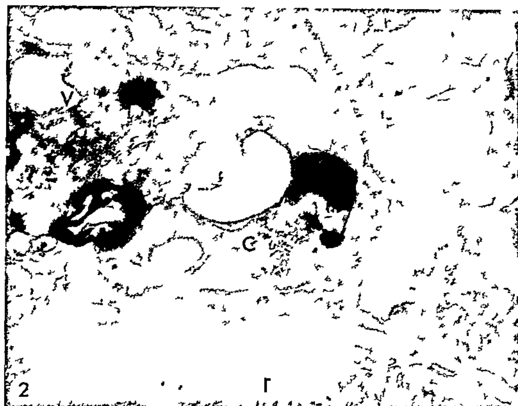
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1 Supported by research grant C–5347 from the *National Cancer Institute U S Public Health Service* and by a grant from the *Sigrid Jusélius Foundation*



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Section of a mouse kidney rudiment after 9 days infection with polyoma virus *in vitro*. A part of a secretory tubule (T) is seen without signs of viral infection. The surrounding mesenchyme shows advanced viral lesions with disintegrating cells, lysosomes (L) and numerous viral particles (V) in nuclei and cytoplasm. Magnification × 6000



F g ⁹
Partial magn ficat on of Fig 1 K dne tul ule (T) seems to l e surrounde l by a basement membrane D type virus part cles (V) are seen in a disintegrat ng me en chymal cell A fev 50 mμ particles are also to l e found in the cytoplasm (C)

were fixe l 3 to 11 lays after infection and prepare l for electron microscopy Cor respond ng ser es with non infected controls v ere made The exper ments w th 7 8 and 9 days infected rud ments were repeate l twice Sections from 20 k dneys representing rud ments from 3 to 11 days subsequent to infection were thoroughly exam ne l In ad lition 12 non nfected k dne s from tl e same la v ere stud ed The total number of cross sections from secretory tul ules v as estimate l at five hundred in the exper mental series and three l undred in the control exam nat ons

RESULTS

Examin ition of the uninfected control l idneys (see Material) re vealed a normal development as described in our earlier studies (*Saxen et al* 1962) A thorough examination of these specimens at magnifications up to 80 000 × revealed no virus lil e particles in the tissue Occasional disintegrating cells were visible and mitoses were frequent

Low power examination of the infected kidneys revealed that the first cytopathic effects appeared 4 days subsequent to infection In kidneys fixed during 7th to 9th day of cultivation all the signs of viral lesions were seen nuclear pycnosis ballooning of the nuclei vacuoliza tion of the cells and finally complete disintegration (Figs 1 and 2) The



Figs 3 and 4

Fig 3 Part of a mesenchymal cell with a cytoplasmic vacuole similar to that seen in Fig 1 (V) 7 days after infection of kidney rudiment with polyoma virus The vacuole seems to be filled with type D virus particles Filamentous structures (F) are also to be seen

Fig 4 Part of the nucleus of a mesenchymal cell in a mouse kidney rudiment 10 days subsequent to infecting with polyoma virus Numerous D type polyoma virus particles can be seen

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POLYOMA VIRUS

2 *Further Studies on the Interaction of Polyoma Virus with Mouse Embyo Cells at Low Temperature*

By

K HELGELAND J JONSEN and O LAHELLE

Received 13 xii 64

In a previous communication *Helgeland, Lahelle & Jonsen* (4) reported that the production of polyoma virus haemagglutinins at 37° C by monolayers of primary mouse embryo cells depends upon the temperature used for the initial cell virus interaction A lower haemagglutination titre was found when the initial cell virus interaction took place at 37° C as compared with that obtained at 22° C and 4° C The interaction at 4° C gave a slightly better yield than that found after interaction at 22° C

The present report deals with further experiments by which to elucidate the mechanism responsible for the observed temperature effect

The production of infectious virus following cell virus interaction at 37° C and 4° C has been measured by means of a plaque assay The percentage of infected cells has also been estimated by infective centre titrations The effect of low temperature interaction in plaque titrations has been investigated In addition the nature of the infectious state produced at the two temperature levels has been explored by application of polyoma virus antiserum

MATERIALS AND METHODS

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Procedure The virus suspension was added at 37° and 4° C respectively

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MATERIALS AND METHODS

Virus A subline[1] of SE polyoma virus was used for all experiments The virus had undergone several passages in monolayer cultures of primary mouse embryo cells The source of virus was infectious tissue culture fluid which had been clarified by low speed centrifugation (10 min at 800 × g)

Cell cultures Primary mouse embryo cells [illegible] medium was changed to Eagle [illegible] 2 per cent horse serum

Infection procedure The virus suspension was added at 37° and 4° C respectively

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MATERIALS AND METHODS

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[illegible] erature The virus suspension was added at 37° and 4° C respectively

[1] Originally obtained from Professor *Herbert Morgan* University of Rochester and designated [illegible] polyoma virus LS VI

to cells grown in prescription bottles The solutions and the cell cultures to be used were equilibrated at the corresponding temperatures The growth medium was removed and the cell layer was washed twice with 7 5 ml of Eagle Hanks medium with 2 per cent horse serum To each cell culture 7 5 ml of virus suspension was added After an interaction time of 3 hours the virus suspension was removed and the cell layer and the adjoining glass surfaces were carefully washed two times with 7 5 ml of Eagle Hanks medium with 2 per cent horse serum Finally 15 ml of the same medium was added and the cultures were incubated at 37° C.

Haemagglutinin assay Extracellular and intracellular haemagglutinins were estimated To release intracellular virus the monolayers were trypsinized the cells spun down at low speed centrifugation and the sedimented cells suspended in phosphate buffered saline of pH 7 2 (PBS) The suspension was then frozen and thawed four times Bound virus was released by treatment with receptor destroying enzyme (RDE) according to *Crawford* (1) Prior to titration the RDE treated suspensions were heated at 56° C for 30 minutes

Haemagglutination (HA) tests were carried out as previously described (*Helgeland Lahelle & Jonsen* (4))

Plaque assay Plaque assay was carried out on secondary mouse embryo cell cultures obtained as follows Confluent primary cultures were treated with 0 25 per cent trypsin in Hanks balanced salt solution for 5–10 minutes at 37° C. The cells were suspended by gentle agitation After low speed centrifugation the cells were suspended in medium and diluted to 0 5 × 10^6 cells per ml 60 × 15 mm tissue culture plastic dishes (Falcon Plastics) were seeded with 2 5 × 10^6 cells in 5 ml of medium (Eagle s minimum essential medium with twofold concentrations of amino acids and vitamins in Earle s balanced salt solution supplemented with 0 5 per cent lactalbumin hydrolyzate and 20 per cent calf serum) The cultures were incubated at 37° C in an atmosphere of 3 per cent CO_2 in air Complete monolayers had formed after incubation for 24 hours The monolayers were washed once with 2 5 ml of Dulbecco s phosphate buffered saline (*Dulbecco & Vogt* (2)) supplemented with 1 per cent horse serum and Eagle s amino acids and vitamins in twofold concentrations (DPBS) The cells were infected at 22° C with virus diluted in the same medium The inoculum was removed after adsorption for 3 hours The plates were overlaid with 7 ml of an agar nutrient mixture containing 2 per cent bacto agar (Difco) Eagle Earle s medium with twofold concentrations of amino acids and vitamins 0 5 per cent lactalbumin hydrolysate and 2 per cent horse serum

After incubation for 4 days the plates were refed with 3 ml of the above agar nutrient mixture Refeeding was repeated on the 8th and 13th day of incubation The last refeeding contained neutral red to give a final concentration of 1 40 000 The number of plaques were counted on the following day

Infective centre assay The method B of *Winocour & Sachs* (11) was employed Monolayers of primary mouse embryo cells grown in prescription bottles were inoculated with virus at 4° and 37° C After adsorption for 3 hours the bottles were washed four times with 7 5 ml of Eagle Hanks medium with 2 per cent horse serum The cells were then suspended by trypsinization and further washed at room temperature in the same medium by two cycles of low speed centrifugation When the cells had been counted and serially diluted in medium 0 1 ml aliquots of the cell dilutions were added to 5 ml of medium in plaque assay dishes which contained confluent monolayers After incubation overnight to allow attachment of the cells to the assay plate monolayers the medium was withdrawn and 7 ml of the plaque assay agar nutrient mixture was added The cultures were subsequently treated according to the method of plaque assay of virus alone

Antiserum Newborn mice less than 24 hours old were inoculated with polyoma virus in cell culture medium After 6 12 months the mice were bled to yield anti [illegible] titrations were made according to *Rowe et al* [illegible] in the range 1/512 1/2048 A single antiserum [illegible]

Prior to treatment with serum the infected monolayers were washed two times with 2 5 ml of cold DPBS to remove unadsorbed virus The cell cultures were then exposed to 0 5 ml antiserum diluted in DPBS After treatment for 1 hour at 4° C. residual serum was removed by four washings with cold DPBS

TABLE 1

Effect of Initial Cell Virus Interaction Temperature on Early Production of Haemagglutinins

Treatment prior to HA titration	Interaction temperature	Extracellular HA on day: HA titre 0	HA titre 1	HA titre 2	Total HA units* 2	Intracellular HA on day: HA titre 2	Total HA units 2
30 minutes at 56°	4°	0 0 0§	0 0 0	64 64 64	4800 4800 4800	256 512 512	2560 5120 5120
	37°	0 0 0	0 0 0	16 16 16	1200 1200 1200	128 128 128	1280 1280 1280
RDE + 30 minutes at 56°	4°	0 0 0	0 0 0	128 128 128	9600 9600 9600	1024 512 512	10240 5120 5120
	37°	0 0 0	0 0 0	32 32 32	2400 2400 2400	256 256 128	2560 2560 1280

* (HA units per ml) × volume

§ Less than 8

At each temperature three prescription bottles were inoculated with 7.5 ml of a virus suspension with a HA titre 1/128. The inoculum was replaced with 15 ml of medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2ml of PBS.

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Antiserum Newborn mice less than 24 hours old were inoculated with polyoma virus in cell culture medium After 6 12 months the mice were bled to yield antiserum Haemagglutination inhibition titrations were made according to *Rowe et al* (10) Inhibition titres were usually in the range 1/512 1/2048 A single antiserum pool was used for all experiments

Prior to treatment with serum the infected monolayers were washed two times with 2 5 ml of cold DPBS to remove unadsorbed virus The cell cultures were then exposed to 0 5 ml antiserum diluted in DPBS After treatment for 1 hour at 4° C, residual serum was removed by four washings with cold DPBS

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	37°	0 0 0	0 0 0	32 32 32	2400 2400 2400	256 256 128	2560 2560 1280

* (HA units per ml) × volume
§ Less than 8
At each temperature three prescription bottles were inoculated with 7.5 ml of a virus suspension with a HA titre 1/128
The inoculum was replaced with 15 ml of medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2ml of PBS

TABLE 2

Effect of Initial Cell Virus Interaction Temperature on Early Production of Infectious Virus

Interaction temperature	*	Extracellular virus on 2 day			Intracellular virus on 2 day		
		EIU plates§	Mean	EIU culture x 10−7	EIU plate	Mean	EIU culture x 10−7
4°	1	40 36 38	38	11 4	70 65 44	60	2 4
	2	48 42 43	44	13 2	58 59,75	64	2 6
	3	37 34 38	36	10 8	[illegible]5 65 68	66	2 6
37°	1	16 21 18	18	5 4	49 59 43	50	2 0
	2	1[illegible] 20	18	5 4	38 39 38	38	1 5
	3	17 16 13	15	4 5	43 36 39	39	1 6

§ The virus suspensions diluted 1 to 10^{-5}
Parallel cultures

RESULTS

Effect of Cell Virus Interaction Temperature on Virus Yield

After cell virus interaction for 3 hours at the respective temperatures the virus suspension was substituted with medium and the incubation continued at 37° C The production of virus haemagglutinins was studied after treatment of the virus suspensions with RDE and infectious virus was estimated in the plaque assay

Table 1 shows the effect of the interaction temperature on the yield of polyoma virus haemagglutinins The yield of both extracellular and intracellular virus in the cultures when the interaction took place at 4° C is significantly higher than the corresponding yields after interaction at 37° C The RDE treatment gives an increased yield of haemagglutinins in both sets of cultures but the difference between the two interaction temperatures still remains The infectious virus titrations (Table 2) also show a beneficial effect of low temperature cell virus interaction on the subsequent production of infectious virus The yield of extracellular haemagglutinins is increased fourfold when the cell virus interaction takes place at 4° C as compared to that obtained after interaction at 37° C and the ratio of extracellular to intracellular haemagglutinins is about 1 2 Table 2 shows that the interaction at 4° C increases the yield of plaque forming units (PFU) about twice, and the ratio of extracellular to intracellular yields of infectious virus is about 3 4 This discrepancy may partly be due to uncertainties in the haemagglutinin determinations

Effect of Cold Treatment of the Cells and the Cell Virus Complex on the Virus Yield

A certain synchronization of EMC virus growth in mouse ascites cells has been obtained by *Martin & Work* (8) when the temperature of the cell virus complex initially was rapidly elevated from 4° to 37° C To investigate whether a similar mechanism played a role in the increased production of polyoma virus after interaction at 4° C cell monolayers were exposed to 4° C for 3 hours before or after an initial cell virus interaction at 37° C for 3 hours Table 3 shows that the cold treatment had no detectable effect on the yield of virus haemagglutinins when compared with the control cultures infected at 37° C

Effect of Cell Virus Interaction Temperature on the Number of Infected Cells

The relationship between the initial cell virus interaction temperature and the number of infected cells was investigated by the infective centre assay The number of infected cells after interaction at 4° C was about twice the number found after interaction at 37° C (Table 4) The input of virus was in the order of 100 PFU per cell and the number of

TABLE 2

Effect of Initial Cell-Virus Interaction Temperature on Early Production of Infectious Virus

Interaction temperature	*	Extracellular virus on 2 day			Intracellular virus on 2 day		
		PFU plates§	Mean	PFU culture x 10⁻⁷	PFU plate	Mean	PFU culture x 10⁻⁷
4°	1	40 36 38	38	11 4	70 65 44	60	2 4
	2	48 42,43	44	13 2	58,59,75	64	2 6
	3	37 34 38	36	10 8	65,65 68	66	2 6
37°	1	16 21 18	18	5 4	49,59,43	50	2 0
	2	16 20	18	5 4	38 39 38	38	1 5
	3	17 16 13	15	4 5	43 36 39	39	1 6

§ The virus suspensions diluted to 10 [illegible]
* Parallel cultures

infected cells, about 10 per cent, is consistent with the values reported by *Winocour & Sachs* (11)

TABLE 4

The Effect of the Cell Virus Interaction Temperature on the Percentage of Infected Cells

Interaction temperature	Number of cells in culture* $\times 10^6$	Number of infected cells§ $\times 10^6$	Percentage of virus—yielding cells
4°	4 43	0 70	15 8
	4 23	0 79	18 7
37°	4 61	0 29	6 3
	4 55	0 37	8 1

* Based on haemocytometer counts
§ Average from three plates

Plaque Counts after Cell Virus Interaction at 37°, 22°, or 4° C

The effect of low temperature interaction in plaque titrations was investigated by counting the number of plaques following an initial cell-virus interaction at 37°, respectively 22° and 4° C.

Plastic dish cultures of secondary mouse embryo cells were equilibrated at 37°, 22° or 4° C and exposed to polyoma virus in 0 5 ml DPBS for 3 hours at the corresponding temperatures The virus dilutions were then removed and the agar overlay was added Table 5 shows that almost identical number of plaques developed in the cultures when the interaction took place at 37° and 4° C, whereas interaction at 22° C increased the number of plaques with about 70–100 per cent Further experiments have shown the increment due to interaction at 22° C to be in the order of 40–100 per cent

TABLE 5

Influence of Temperature on the Initial Cell Virus Interaction in Plaque Assay

Interaction temperature	Experiment no I			Experiment no II		
	PFU plate	Mean	Per cent	PFU plate	Mean	Per cent
4°	46 56 61	54	110	31,28 28	29	85
22°	78 88 84	83	169	74 63 74	70	206
37°	44 46 58	49	100	37 38 26	34	100

Experiment I and II were performed with different virus preparations

Sensitivity of the Cell Virus Complex to Antiserum

The effect of antiserum on the infectious state produced by cell virus interaction at 4° and 37° C was investigated Monolayers in plastic dishes were equilibrated at 4° or 37° C and exposed to a suspension of polyoma virus in 0 5 ml DPBS After interaction for 3 hours at the respective temperatures the monolayers were rinsed free of unattached

TABLE 3

Effect of Exposure to 4° C before or after the Initial Cell Virus Interaction at 37° C on the Production of Virus Haemagglutinins

Pre-treatment at 4° C	Cell-virus inter-action temp	Post-treatment at 4° C	Extracellular HA on day				Intracellular HA on day	
			HA titre			Total HA units*	HA titre	Total HA units
			2	3	5	5	3	5
	4°		32 64 64§	64 128 128	128 256 256	9600,19200 19200	1024 1024 1024	10240,10240 10240
	37°	3 hours	8 8 16	32 32 64	64,128 128	4800 9600 9600	512 512 1024	5120 5120 10240
3 hours	37°		8 16 16	32 32 32	128,128 128	9600 9600 9600	256 512 512	2560 5120 5120
	37°		16 16 16	32 32 64	64 128 128	4800 9600 9600	512 512 512	5120 5120 5120

* (HA units per ml) × volume

§ HA titres of three parallel cultures

Each prescription bottle was inoculated with 7.5 ml of a virus suspension with a HA titre 1/512

The inoculum was replaced with 15 ml medium for virus growth. Intracellular HA was estimated after freezing and thawing the cells in 2 ml PBS

infected cells, about 10 per cent, is consistent with the values reported by Winocour & *Sachs* (11).

TABLE 4

The Effect of the Cell Virus Interaction Temperature on the Percentage of Infected Cells

Interaction temperature	Number of cells in culture $\times 10^6$	Number of infected cells§ $\times 10^6$	Percentage of virus—yielding cells
4°	4.43	0.70	15.8
	4.23	0.79	18.7
37°	4.61	0.29	6.3
	4.55	0.37	8.1

* Based on haemocytometer counts
§ Average from three plates

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Plastic dish cultures of secondary mouse embryo cells were equilibrated at 37°, 22° or 4° C and exposed to polyoma virus in 0.5 ml DPBS for 3 hours at the corresponding temperatures. The virus dilutions were then removed and the agar overlay was added. Table 5 shows that almost identical number of plaques developed in the cultures when the interaction took place at 37° and 4° C, whereas interaction at 22° C increased the number of plaques with about 70–100 per cent. Further experiments have shown the increment due to interaction at 22° C to be in the order of 40–100 per cent.

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virus with cold DPBS and 0.5 ml antiserum diluted in DPBS was added. After exposure to antiserum for 1 hour at 4° C, the monolayers were washed four times with cold DPBS and agar nutrient mixture was added. As controls served monolayer cultures in which antiserum treatment was replaced with a similar treatment with medium alone. From three dishes at each temperature the virus inoculum was removed and the agar overlay added without any preceding treatment.

TABLE 6

Influence of Antiserum on Polyoma Virus Attached to Mouse Embryo Cell Monolayers Grown in Plastic Dishes

Interaction temperature	Treatment of cell monolayers after virus attachment	PFU/plate	Mean	Per cent neutralization of adsorbed virus
	No treatment	110 114 106	110	
4°	Treated with diluting medium	74 68 84	75	0
	Treated with antiserum diluted 10^{-3}	46 55,64	55	27
	10^{-2}	14 10,14	13	83
	10^{-1}	9 6 2	6	92
	No treatment	108 109,118	112	
37°	Treated with diluting medium	112,105,112	110	0
	Treated with antiserum diluted 10^{-3}	92 93 81	89	19
	10^{-2}	76 88 85	83	25
	10^{-1}	58 60 66	61	45

TABLE 7

Influence of Normal Serum on Polyoma Virus Attached to Mouse Embryo Cell Monolayers Grown in Plastic Dishes

Interaction temperature	Treatment of cell monolayers after virus attachment	PFU/plate Mean§	Per cent neutralization of adsorbed virus
	Treated with diluting medium	78	0
4°	Treated with normal serum diluted 10^{-1}	82	0
	Treated with antiserum diluted 10^{-1}	9	88
	Treated with diluting medium	108	0
37°	Treated with normal serum diluted 10^{-1}	113	0
	Treated with antiserum diluted 10^{-1}	48	56

§ Average of three plates

Table 6 shows that there is a neutralizing effect of antiserum which decreases when serum is diluted. The virus in the complexes produced at 4° and 37° C are both sensitive to antiserum but to different degrees. With 10 per cent antiserum 92 per cent of the virus adsorbed at 4° C is neutralized as compared with only 45 per cent after cell virus inter-

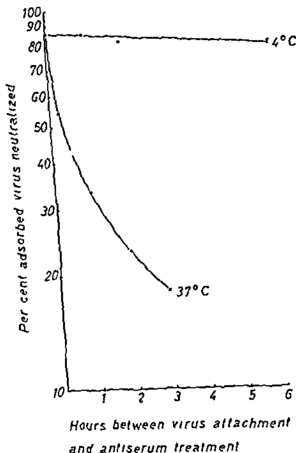


Fig 1

action at 37° C The different sensitivity is less pronounced when 0 1 per cent antiserum is used

When virus was treated with 0 1 per cent antiserum for 1 hour at 4 C prior to infection of cell monolayers no plaques appeared

The decreased plaque counts in cultures washed with medium after cell virus interaction at 4° C indicate that some of the virus was removed by the washings Such a loss was not found with cultures treated with virus at 37° C

Table 7 shows that normal mouse serum has no neutralizing effect

Fig 1 reveals that polyoma virus in contact with mouse embryo cell monolayers became progressively unavailable for neutralization by antiserum at a temperature dependent rate In this experiment the initial cell virus interaction took place at 4° C and after 2 hours the inoculum was removed by two washings with cold DPBS Some of the cultures were then transferred to 37° C and DPBS was added at the

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	10^{-1}	9,6 2	6	92
37°	No treatment	108 109,118	112	
	Treated with diluting medium	112 105 112	110	0
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fore reduced and a smaller number of plaques following cell virus interaction at 4° and 22° C would be expected. When this expectation does not come through it is in support of the assumption that the proportion of hits leading to a permanent preinfectious bond—the collision efficiency—is higher at low temperatures. The increased yield of plaques following cell virus interaction at 22° C might be due to a relatively low viscosity combined with a partial retention of the bond stability at this temperature. *Crawford* (1) found no increase in the plaque count when the temperature used for the initial cell polyoma virus interaction was reduced from 37° to 22° or 4° C. On the other hand, Crawford's experiment also indicated an increased stability of the cell virus bond at low temperatures which counteracted the increased viscosity. The definite answer to this problem must be sought in kinetic experiments. Prior to such experiments we have investigated the nature of the infectious state produced at 4° or 37° C by application of antiserum. We found a pronounced inhibitory effect of antiserum on the early cell virus complex established at 4° C. This has undoubtedly its basis in a reaction of the antiserum with the viral antigen as normal serum had no effect.

Whereas the virus which is bound to the cell at low temperature is sensitive to antiserum the penetration, *i e* the progressive immunity of adsorbed virus to inactivation by external applied antiserum, is greatly accelerated at *37° C*. *Fig 1* shows that penetration starts without any delay when transferred to 37° C. After 30 minutes at this temperature about 50 per cent of virus has become resistent to antiserum. By con-

... therefore possible that antiserum had some neutralizing effect also *in later* stages of penetration. It can not be excluded, however, that some virus exhibit a lag period before penetration starts and that virus *during this lag* is accessible to the neutralizing effect of antiserum. The *resistent state* of virus in the penetration could be explained by assuming a *progressive binding* of virus so as to cover sites reactive to antibody or by some mechanism providing physical inaccessibility. At some time in the penetration process one would however expect separation of *virus DNA from* the antigenic protein coat. When such separation has occurred the *infectious DNA* has escaped the action of antibody and virus emerges in the resistent state.

The results obtained *so far in* the described experiments with antiserum treatment of *polyoma* virus—mouse embryo cell complexes are similar to those reported from *experiments* with influenza virus (*Ishida & Ackermann* (6)), vaccinia virus (*Postlethwaite* (9)) and poliovirus (*Holland & McLaren* (5), *Mandel* (7)).

respective temperatures After varying periods of incubation at 37° or 4° C the medium was removed and the cultures were treated at 4° C *with 10 per cent antiserum for 1 hour*

The figure shows that 13 per cent of the bound virus is resistent to antiserum at zero time after cell-virus interaction at 4° C The small increase in the amount of antiserum resistent virus after further in cubation for 6 hours at 4° C is hardly significant At 37° C, however, 50 per cent of the virus became antiserum resistent after about 30 minutes After 3 hours at this temperature about 80 per cent of the input virus had become resistent

The results indicate that the adsorbed virus is initially held at the cell surface where it is susceptible to antiserum, but at 37° C penetrates beyond the sphere of antiserum influence

DISCUSSION

The present results confirm earlier experiments which indicated that the interaction of polyoma virus with primary mouse embryo cell cultures at 4° C leads to a production of virus haemagglutinins, by sub sequent incubation of the infected cells at 37° C, which is higher than that obtained when the initial interaction takes place at 37° C

The infective centre titrations showed that the most likely explanation for this temperature effect is found in an increased number of in fected cells by interaction at 4° C compared with that obtained at 37° C

Cold treatment of the cells alone had no detectable effect when the cells were subsequently infected at 37° C Nor had a similar cold treat ment of the cell virus complex established at 37° C any effect It is therefore reasonable to assume that the explanation of the higher yield of virus after interaction at low temperature is found in an effect on the process of virus adsorption, and not on later steps in the infection procedure

The plaque experiments gave results in apparent disagreement with those obtained in the production experiments as the plaque assay gave about the same number of plaques by interaction at 4° and at 37° C, respectively An interaction temperature of 22° C in the plaque assay gave, however, a 40 100 per cent increase in the number of plaques The explanation of the discrepancy to occur when two different parameters, the yield of haemagglutinins and the plaque assay are used may be sought in the different methods employed The plaque assay is per formed with a very low multiplicity of infection and a small volume of virus dilution whereas the production experiments were performed with a multiplicity of infection in the order of 100 plaque forming units per cell and a relatively large volume of the virus suspension At low temperatures the Brownian movements of suspended virus particles are decreased because of the higher viscosity of the suspending medium The number of particles colliding with the cells per time unit is there-

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THE PASSAGE OF PENICILLIN INTO THE CEREBROSPINAL FLUID AFTER INTRACISTERNAL INJECTION OF DEAD BACTERIA OR BACTERIAL TOXINS

An Experimental Investigation in Rabbits

By

ARNE LITHANDER

Received 17 vii 64

The passage of chemotherapeutic preparations through the blood cerebrospinal fluid barrier into the cerebrospinal fluid (C S F) is of great importance in bacterial infections in the central nervous system Experimental investigations of this problem are few in number *Lithander & Lithander* (1) found that in experimentally produced staphylococcal meningitis in rabbits the passage of benzyl penicillin into the C S F was proportionate to the degree of the meningitis In relation to the clinical symptoms the penetration of the barrier appeared to occur at different times and to different extents in different infections (2) Infections with β streptococci appeared to break down the blood C S F barrier sooner than infections with α and β streptococci, pneumococci meningococci and staphylococci In addition β-streptococci seemed to have a greater effect on the barrier than the other bacteria

The question arose in this connection whether bacterial toxins might be of significance to the passage of penicillin through the blood C S F-barrier The present paper represents an experimental investigation in rabbits concerning this question and also the question of the influence of dead bacteria

MATERIAL AND METHODS

The investigation was conducted on rabbits weighing approximately 2 5 kg The animals were given intracisternal injections of bacterial toxins or of dead bacter[illegible]

[illegible] staphylococci (strain 209) β streptococci Group A Type 1 (one strain) and meningococci (one strain) An intracisternal injection of 0 5 ml of each

[1] The penicillin in the present investigation was kindly supplied by AB Kabi Stockholm Sweden

SUMMARY

The amount of polyoma virus produced at 37° C depends upon the temperature used for the initial cell-virus interaction The yield of both virus haemagglutinin and infectious virus increased 2–4 fold following an initial cell-virus interaction at 4° C compared with that obtained after interaction at 37° C

With a multiplicity of infection in the order of 100 PFU per cell about 17 per cent of the cells were infected following an initial cell-virus interaction at 4° C as compared with about 7 per cent infected cells at 37° C.

In the plaque assay almost identical number of plaques were obtained following an initial cell-virus interaction at 4° or 37° C When this interaction took place at 22° C the number of plaques increased by 40–100 per cent.

Antiserum treatment of the infectious state produced at 4° or 37° C showed that the cell-virus complex established at 4° C is sensitive to the action of antiserum At 37° C the virus became progressively inaccessible to antiserum at a time dependent rate, possibly due to penetration of the virus into the cell.

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The Bacteriological Department of Serafimerlasarettet Stockholm
(Head Arne Lithander M D)

THE PASSAGE OF PENICILLIN INTO THE CEREBROSPINAL FLUID AFTER INTRACISTERNAL INJECTION OF DEAD BACTERIA OR BACTERIAL TOXINS

An Experimental Investigation in Rabbits

By

ARNE LITHANDER

Received 17 vii 64

The passage of chemotherapeutic preparations through the blood cerebrospinal fluid barrier into the cerebrospinal fluid (C S F) is of great importance in bacterial infections in the central nervous system Experimental investigations of this problem are few in number *Lithander & Lithander* (1) found that in experimentally produced staphylococcal meningitis in rabbits the passage of benzyl penicillin into the C S F was proportionate to the degree of the meningitis In relation to the clinical symptoms, the penetration of the barrier appeared to occur at different times and to different extents in different infections (2) Infections with β streptococci appeared to break down the blood C S F barrier sooner than infections with α- and β streptococci, pneumococci, meningococci and staphylococci In addition, β streptococci seemed to have a greater effect on the barrier than the other bacteria

The question arose in this connection whether bacterial toxins might be of significance to the passage of penicillin through the blood C S F-barrier The present paper represents an experimental investigation in rabbits concerning this question and also the question of the influence of dead bacteria

MATERIAL AND METHODS

The investigation was conducted on rabbits weighing approximately 2 5 kg The animals were given intracisternal injections of bacterial toxins or of dead bacteria under anaesthesia with nembutal The bacterial suspensions were prepared with [illegible]

[1] The penicillin in the present investigation was kindly supplied by AB Kabi Stockholm Sweden

SUMMARY

The amount of polyoma virus produced at 37° C depends upon the temperature used for the initial cell-virus interaction. The yield of both virus haemagglutinin and infectious virus increased 2–4 fold following an initial cell-virus interaction at 4° C compared with that obtained after interaction at 37° C.

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TABLE 1

Penicillin Concentration in Plasma, Cerebrospinal Fluid (C.S.F.) and Brain and Concentration of White Corpuscles in Cerebrospinal Fluid

Group	N	Plasma (1)		C.S.F.		Brain		% Quotient	White Corpuscles	
		M ± ϵ_M	σ	M ± ϵ_M	σ	M ± ϵ_M	σ	C.S.F./P	M ± ϵ_M	σ
Staphylococcic toxin	10	2.45 ± 0.10	0.36	0.87 ± 0.06	0.18	0.94 ± 0.05	0.16	2.6	2.82 ± 0.08	0.26
Endotoxin	10	2.56 ± 0.08	0.26	1.14 ± 0.06	0.18	1.14 ± 0.05	0.16	3.8	3.33 ± 0.13	0.36
Scarlatinal toxin	12	2.43 ± 0.06	0.20	1.17 ± 0.05	0.17	1.05 ± 0.04	0.15	5.5	2.40 ± 0.06	0.22
Dead staphylococci	10	2.67 ± 0.08	0.26	0.97 ± 0.06	0.18	1.12 ± 0.06	0.19	2.0	2.66 ± 0.05	0.17
Dead meningococci	10	2.38 ± 0.06	0.18	1.20 ± 0.06	0.17	1.00 ± 0.05	0.15	6.7	4.39 ± 0.09	0.27
Dead β streptococci	11	2.70 ± 0.14	0.47	1.59 ± 0.13	0.44	1.26 ± 0.08	0.27	7.8	3.46 ± 0.18	0.59
Controls*	32	2.33 ± 0.05	0.28	0.68 ± 0.04	0.20	0.83 ± 0.04	0.24	2.2	1.88 ± 0.22 (N = 19)	0.96

Penicillin concentrations expressed as log (100 × mean of I.U. per ml)
Concentrations of white corpuscles expressed as log (mean number of corpuscles per ml)
* The levels for the controls were obtained from an earlier investigation (1)

suspension of dead bacteria was made following removal of the same amount of C S F In the case of the dead staphylococci 20 million bacteria were injected i e double the dose which produced meningitis in the experiments with live bacteria (1) Dead β streptococci and meningococci were injected in doses of 400 to 2 000 and 3 000 to 5 000 million bacteria, respectively, i e in approximately the same amounts which produced meningitis in the experiments with live bacteria (2) The bacterial toxins used were staphylococcus toxin (strain Walker) scarlatinal toxin and endotoxin[1] Injections of 0 5 ml of the toxins or dilutions thereof were given The controls were the same as in an earlier investigation (1)

Twenty-four hours later the clinical condition of the injected rabbits was studied with particular reference to meningeal symptoms, 20 000 units of benzyl penicillin[2] per kg body weight were then administered intravenously Cisternal puncture and removal of C S F were carried out 30 minutes after the injection of penicillin In rapid succession thereafter, a sample of blood was taken from the jugular vein the animal was allowed to bleed to death and the brain was removed for determination of penicillin content and for microscopic examination The techniques used for these measures and for the penicillin determination in the C S F , plasma and brain substance were the same as in earlier investigations (1, 2)

RESULTS

The injections of dead bacteria and toxins produced no signs of meningitis in the majority of cases At the same time there were hints of symptoms of meningitis in a few cases and actual mild signs in one case The concentration of white corpuscles in the C S F (Table 1) was slightly higher in the experiments with dead staphylococci, staphylococcus toxin and scarlatinal toxin than in the controls (1), in which physiological saline solution was injected Endotoxin and dead β-streptococci gave a moderate, and dead meningococci a high, concentration of white corpuscles in the C S F The concentration of red corpuscles in the C S F was very low in all the series

The *penicillin concentration in plasma* (Table 1) in the experiments with dead staphylococci and β-streptococci was slightly higher than in the other series, which were approximately the same in this respect

The *penicillin concentration in C S F* (Table 1 and Figs 1 and 2) was considerably higher in the dead β-streptococci series than in the other five series The penicillin concentrations were largely the same in the series with dead staphylococci and with staphylococcic toxin as in the controls in an earlier investigation (1) they were slightly higher in the series with dead meningococci, endotoxin and scarlatinal toxin

The penicillin concentrations in C S F were related to the penicillin concentrations in plasma in all of the series The correlations were significant with exception of the series with dead staphylococci In this series the correlation was uncertain

The ratio $\frac{\text{penicillin concentration in C S F}}{\text{penicillin concentration in plasma}} \left(\frac{\text{C S F}}{\text{P}}\right)$ (Table 1) was highest in the experiments with dead β-streptococci and meningococci

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[1] The endotoxin used in the experiments was Bacto lipopolysaccharide (E coli 0111 B 4) from the Difco Laboratories Detroit Mich U S A

[2] Hereinafter the term benzyl penicillin will be abbreviated to penicillin

TABLE 1

Penicillin Concentration in Plasma Cerebrospinal Fluid (C S F) and Brain and Concentration of White Corpuscles in Cerebrospinal Fluid

Group	N	Plasma (P)		C S F		Brain		% Quotient	White Corpuscles	
		$M \pm \varepsilon_M$	σ	$M \pm \varepsilon_M$	σ	$M \pm \varepsilon_M$	σ	C S F / P	$M \pm \varepsilon_M$	σ
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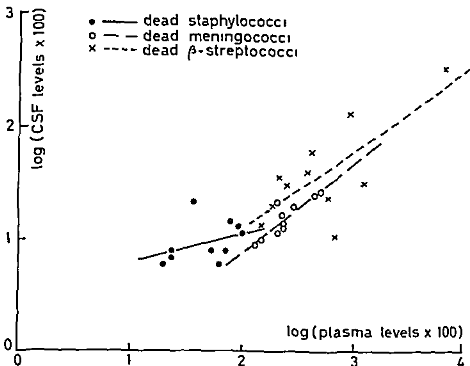


Fig 1

Penicillin concentrations in plasma and C S F in rabbits after intracisternal injections of dead staphylococci meningococci and β streptococci

$\frac{\text{C S F}}{\text{P}}$ in these experiments was almost the same as in those with live staphylococci, α- and γ-streptococci, meningococci and pneumococci (2), when the clinical signs of meningitis were either absent or only suggested or mild In experiments with dead β-streptococci and men-

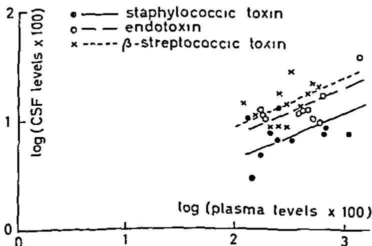


Fig 2

Penicillin concentrations in plasma and C S F in rabbits after intracisternal injetions of staphylococcic scarlatinal and endotoxin

ingococci, $\frac{C\ S\ F}{P}$ was clearly higher than in the series with dead staphylococci and with staphylococcus toxin and endotoxin. In the three last mentioned series $\frac{C\ S\ F}{P}$ was largely the same as after the same dose of penicillin in control rabbits given intracisternal injections of physiological saline solution. In the series with scarlatinal toxin $\frac{C\ S\ F}{P}$ was somewhat lower than in the experiments with dead β streptococci and meningococci.

Any relationship between the penicillin concentration in C S F and the concentration of white and red corpuscles was not seen in any of the series.

The *penicillin concentration in the brain* was almost invariably low in all of the series and, on the whole, independent of the penicillin concentration in plasma.

Microscopic examination of specimens of the meninges removed from the rabbits showed only slight changes and scarcely any signs of inflammation. No difference could be detected between the various groups of rabbits injected with dead bacteria or bacterial toxins.

DISCUSSION

As a rule the intracisternal injections of dead bacteria or bacterial toxins did not give rise to signs of meningitis. However, a few of the rabbits injected with dead β streptococci revealed hints of such signs. The concentration of white corpuscles in the C S F was considerably increased in rabbits injected with endotoxin and with dead β streptococci or dead meningococci. In the other series, the concentration was only slightly higher than levels observed (1) in control rabbits given injections of physiological saline solution only. The higher concentration observed, especially in the experiments with dead meningococci and β streptococci may possibly be related to the fact that these bacteria were injected in much greater quantities than the ones used in the experiments with dead staphylococci. As a result, an aseptic irritation may have been produced in the meninges in the two first mentioned series.

The penicillin concentration in C S F in the experiments with dead β streptococci was clearly increased and was higher than in the other five series. The dead β streptococci had an effect on the blood C S F barrier in this respect which resembled that achieved with live β streptococci in experiments on rabbits in which no clinical signs of meningitis were present (2). The effect of other agents used in this st[illegible]
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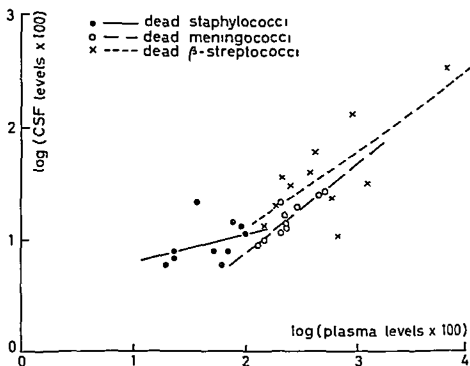


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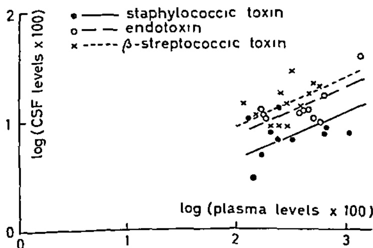


Fig 2
Penicillin concentrations in plasma and C S F in rabbits after intracisternal injections of staphylococcic scarlatinal and endotoxin

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The National Bacteriological Laboratory and the Department of Virus Research, Karolinska Institutet, School of Medicine, Stockholm, Sweden

SMALLPOX VACCINATION STUDIES WITH SERIAL DILUTIONS OF VACCINE

I. *Primary Vaccination and Revaccination in Human Adults*

By

J. ÅKE ESPMARK

Received 17.vii.64

The trivial fact that the rate of takes after smallpox vaccination depends both on vaccine potency and on the immunity status of the vaccinees is more or less contained in the definitions of "potency" and "immunity". Quantitatively, however, such relationships have been only incompletely investigated until the last few years. Problems adequate in this connection concern for instance the distribution of take frequencies over a range of defined vaccine potencies, or the numerical estimation of the relative susceptibility of immune and non-immune populations.

It seems that these questions did not really attract attention within the smallpox vaccine field until relatively lately, when similar problems had already been formulated and experimental methods had been elaborated within other branches of microbiology.

The first reliable attempt to study the response distribution in humans after application of smallpox vaccines of a wide range of potencies was made by *Cockburn et al.* (4). A series of vaccines with decreasing egg pock count titers gave falling take percentages which seemed to fit a normal sigmoid response curve. The 50 per cent infective dose was estimated at 10^{5} pock forming units of virus per ml. Data below the 75 per cent take level were, however, rather limited in this study. Since potencies were estimated through titrations, there was some error (although relatively small) also in the potency variable.

In studies on vaccinia virus infection in rabbit skin, *Parker* (23) found that the dose response relationship followed a one-hit Poisson distribution, which is also sigmoid shaped, though slightly assymmetrical, and he thus verified the assumption that infection could be elicited by one infectious particle.

The influence of previous immunity on the outcome of revaccination was investigated by *Gins* (16) and later more thoroughly by *Broom* (3), *Vasilyukova et al.* (22) and by *Cross* (5). They all found gradually

cisternal injections of physiological saline solution, which might indicate that the blood-C S F barrier was affected although there had not been any infection in the meninges However, only in experiments with dead β-streptococci and meningococci and with scarlatinal toxin was the ratio $\frac{C\ S\ F}{P}$ appreciably higher after the same dose of penicillin than in control rabbits given injections of physiological saline solution (1) This shows that in this investigation only these three agents exerted a real influence on the blood-C S F barrier with regard to the passage of penicillin

SUMMARY

1 An investigation was made of the passage into the C S F of intravenous penicillin following intracisternal injection of dead bacteria and of bacterial toxins

2 The injection of dead β-streptococci and meningococci and of scarlatinal toxin led to an intensification of the passage of penicillin through the blood-C S F barrier which resembled the influence exerted by live bacteria The effect of scarlatinal toxin was somewhat less pronounced

Dead staphylococci, staphylococcus toxin, and endotoxin had no effect on the blood-C S F in this respect

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The influence of previous immunity on the outcome of *revaccination* was *investigated* by *Gins* (16) and later more thoroughly by *Broom* (3), *Maslyukova et al.* (22) and by *Cross* (5). They all found gradually

increasing vaccination success rate with increasing time interval since the last vaccination

Neither of these studies included detailed specifications as to vaccine potency

In the vaccination study to be reported on here, serial dilutions of smallpox vaccines were utilized to reduce the error of the relative "dose" The aims of the experiments were.

(a) to investigate the relation between vaccine potency (dose) and the proportion of positive reactions (quantal response), covering a wide take frequency range, *i e* to determine the shape and slope of the dose-response curve,
(b) to study if vaccines of different origin (calf lymph, egg vaccine, tissue culture vaccine) would give essentially similar dose response relationships,
(c) to compare the dose-response behaviour of various human populations differing in immunity status

The essential results of this study were summarized in a preliminary report in 1961 (9) A detailed account will be given in the present communication

A technique similar to that utilized in this study has been recently used also by *Polak et al* (24) in attempts to compare two smallpox vaccines in primary vaccinations

MATERIAL AND METHODS

THE VACCINEES The group under study consisted of male conscripts 19 21 years of age, entering their military service The history of previous vaccination was recorded through questions and observations of vaccination scars With respect to vaccination history the following three categories were distinguished

(1) persons not previously vaccinated (primary vaccinations)
(2) those vaccinated in infancy or childhood *i e* more than 10 years ago (late revaccinations)
(3) people previously vaccinated with supposedly positive results 1 3 years ago (early revaccinations)

Most of the conscripts (about 90 per cent) belonged to group 2 and somewhat less than 10 per cent had not been vaccinated before

Recently vaccinated (group 3) were few variability was high and results thus difficult to evaluate statistically They were included however in order to give an idea of the vaccination response in a population with a relatively high degree of immunity This immunity may have been somewhat over estimated since questioning was the only source of information about the last vaccination in this group

VACCINES Four different vaccines were used one glycerinated calf lymph vaccine (C1 8/51) produced in the conventional way one tissue culture vaccine (Tc 341) derived from suspended bovine embryonic skin culture according to *Wesslen* (28) and two chorio allantoic membrane vaccines (F 13 and I 29) produced as described elsewhere (10) From each vaccine logarithmic dilution series (2 fold or 3 16 fold) were prepared using 0 3 per cent agar in distilled water with 2 per cent normal heated horse serum as a diluent The vaccine dilutions were dispensed in single dose plastic ampoules (15), which were stored in rubber stoppered glass tubes at —25° C and brought to the vaccination clinic on dry ice in thermos bottles

Rabbit test a modification of the Calmette Guerin (18) pock enumeration method in scarified skin was used The titer was first roughly estimated in an exploratitive titration and then three 2 fold dilutions were inoculated one of which was expected to give 3 30 pocks per 0 3 ml inoculum Results from rabbit tests will be given only as crude pock counts for comparison

Egg titrations were performed as pock counting on the dropped chorioallantoic membrane (CAM) of 12 day old chick embryos following essentially the method of Westwood *et al* (29) From preliminary titrations a dilution was calculated which would give 5 20 pocks (preferable about 10) per 0 2 ml inoculum In the final titration three consecutive 2 fold dilutions were inoculated in 12 eggs each and the titer as the number of pock forming units (pfu) per ml was calculated from the average pock count of one dilution

Tissue culture tube titrations were made in stationary (standing) 100 × 13 5 mm

ml of 1 per cent rooster erythrocytes had been added to each tube At the reading which was made macroscopically positive tubes showed a typical agglutination bottom pattern or sharp rounded focal hemadsorption Twenty uninoculated control tubes were included in each titration Titers (log ID_{50} per ml) were calculated according to Kärber (20) Sources of error and variability are discussed in detail in separate reports (11 12)

VACCINATIONS The lateral subdeltoid region of the left arm was cleaned with an

[illegible] was used for each site All inoculations in this study were performed by the author

Readings of vaccination results were made twice Most readings were made 3 and 7 days after vaccination In some cases other reading times were used as recorded below under the separate experiments

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THE STATISTICAL TREATMENT of the quantal response data including probit analysis is the subject of a separate paper (13) to which reference will be made in so far as computed statistics are needed In the present report results will be illustrated mainly through tables and graphical methods

DEFINITION OF SOME TERMS USED IN THE TEXT *Dose* The term "dose" denoting the independent variable of the dose response [illegible] will refer to units of virus concentration [illegible] dilution or as logarithm of relative concen[illegible] namely that only an un known fractio[illegible] tually inoculated With due allowance for [illegible] inadequacy the term dose" will be used for the sake of convenience

Response Unless otherwise specified response refers to clinically observable local signs following vaccination This is mentioned to avoid any confusion with the immunological meaning of the term

Quantal response This expression [illegible]

increasing vaccination success rate with increasing time interval since the last vaccination

Neither of these studies included detailed specifications as to vaccine potency

In the vaccination study to be reported on here, serial dilutions of smallpox vaccines were utilized to reduce the error of the relative "dose" The aims of the experiments were

(a) to investigate the relation between vaccine potency (dose) and the proportion of positive reactions (quantal response), covering a wide take frequency range, *i e* to determine the shape and slope of the dose-response curve,
(b) to study if vaccines of different origin (calf lymph, egg vaccine, tissue culture vaccine) would give essentially similar dose response relationships,
(c) to compare the dose-response behaviour of various human populations differing in immunity status

The essential results of this study were summarized in a preliminary report in 1961 (9) A detailed account will be giveen in the present communication

A technique similar to that utilized in this study has been recently used also by *Polak et al* (24) in attempts to compare two smallpox vaccines in primary vaccinations

MATERIAL AND METHODS

THE VACCINEES The group under study consisted of male conscripts 19–21 years of age entering their military service The history of previous vaccination was recorded through questions and observations of vaccination scars With respect to vaccination history the following three categories were distinguished

(1) persons not previously vaccinated (primary vaccinations)
(2) those vaccinated in infancy or childhood *i e* more than 10 years ago (late revaccinations)
(3) people previously vaccinated with supposedly positive results 1 3 years ago (early revaccinations)

Most of the conscripts (about 90 per cent) belonged to group 2 and somewhat less than 10 per cent had not been vaccinated before

Recently vaccinated (group 3) were few variability was high and results thus difficult to evaluate statistically They were included however in order to give an idea of the vaccination response in a population with a relatively high degree of immunity This immunity may have been somewhat over estimated since questioning was the only source of information about the last vaccination in this group

VACCINES Four different vaccines were used one glycerinated calf lymph vaccine (C I 8/51) produced in the conventional way one tissue culture vaccine (T c 341) derived from suspended bovine embryonic skin culture according to *Wesslen* (28) and two chorio allantoic membrane vaccines (F 13 and F 29) produced as described elsewhere (10) From each vaccine logarithmic dilution series (2 fold or 3 16 fold) were prepared using 0 3 per cent agar in distilled water with 2 per cent normal heated horse serum as a diluent The vaccine dilutions were dispensed in single dose plastic ampoules (15), which were stored in rubber stoppered glass tubes at —25° C and brought to the vaccination clinic on dry ice in thermos bottles

TABLE 1

Results of Laboratory Potency Tests on Smallpox Vaccines Used in the Vaccination Trial

Vaccine	Stock suspension of vaccine diluted	Test in scarified rabbit skin: Number of pocks in 2 rabbits inoculated with 0.3 ml of the respective dilution series of stock suspension — 1:100	1:500	1:1000	1:2000	1:4000	EID50/ml in chick embryos (Each value average of 3 titrations with 12 eggs per dil.)	ID50/ml in monkey kidney tissue culture tubes (Each value average of 2 titrations with 10 tubes per dil.)
CI 851	1:60	+++*	14	10	7		6.6×10^{7}	$10^{8.0}$
		+++	33	11	3		5.5×10^{7}	$10^{7.95}$
Fe 341	1:2			27	7	2	3.5×10^{6}	$10^{6.95}$
				30	42	1	3.1×10^{6}	
1 13	1:20	25	13	3	2		3.9×10^{7}	$10^{8.1}$
		26	14	7	4		3.3×10^{7}	$10^{8.0}$
1 29	1:100	40	15	5	0		7.6×10^{7}	$10^{8.15}$
		18	5	0	0			

* > 50 pocks

Late revaccination meaning revaccination more than 10 years after the last vaccination and *early revaccination* i e revaccination 1–3 years after the last successful vaccination will be used frequently in the text without details of vaccination history being repeated each time

RESULTS

Vaccine Titers

Titer values for the four different vaccines undiluted, obtained by the three laboratory methods mentioned, are shown in Table I Only some brief comments on the titer values will be made here The tissue culture titers are rather similar (i e about $10^{8.0}$ ID_{50} per ml) for the calf lymph C I 8/51 and the two egg vaccines, whereas the tissue culture vaccine is more than one $\log_{10}$ lower in titer Approximately the same titer relationships between vaccines hold also for egg-titers The latter are probably somewhat underestimated since variability in individual membrane pock-counts was large compared with that from some other laboratories with longterm experience from the egg-method (*e g Westwood et al (29)*) This defect, probably due to a relatively high frequency of subnormal pock-counts from technically injured eggs, may be expected to have reduced the sensitivity (*i e* the height of titer values), whereas precision would be affected to a smaller extent as adequate numbers of eggs were used

The potencies af vaccines C I 8 51, E 13 and E 29 undiluted thus correspond approximately to the requirements proposed by the W H O (30)

1 *Preliminary Experiments*

a *The importance of reading time Transient vesiculation* In explorative revaccinations with serial 10 fold dilutions of vaccine and readings after 2, 3, 5 and 7 days it was noted that vesicle formation started relatively early (after 2 days) with high concentrations of vaccine and later with less potent vaccines On very rare occations vesicles appeared after more than 5 days This is corroborated by findings in a separate dose-incubation time study reported elsewhere (14) It was considered, therefore, that few takes would escape observation if the final reading was made 5 days after inoculation In fact the majority of the reactions in the following trial were read after 7 days

On the other hand, on some occasions vesicles formed at 2 days had vanished at the 5 day reading In a number of persons with pronounced vaccinoid reactions to the most potent vaccine dilution such transient vesiculation was noted on sites inoculated with weaker dilutions Relevant in this connection is also the observation reported by *Groth* (17) that, in some cases, different "types" of revaccination reactions were seen simultaneously in the same person inoculated at several sites *Broom* (3) and *Benenson* (2) reported that early reactions ("allergic responses") could be elicited also with killed vaccine

TABLE 1

Results of Laboratory Potency Tests on Smallpox Vaccines Used in the Vaccination Trial

Vaccine	Stock suspension = vaccine diluted	Test in scarified rabbit skin: Number of pocks in 2 rabbits inoculated with 0.1 ml of the respective dilution series of stock suspension — 1:100	1:500	1:1000	1:2000	1:4000	Pfu/ml in chick embryos (Each value average of 5 titrations with 12 eggs per dil.)	ID_{50}/ml in monkey kidney tissue culture tubes (Each value average of 2 titrations with 10 tubes per dil.)
CL 8/51	1:50	+++* +++	14 31	10 11	7 3		6.6×10^7 5.5×10^7	$10^{8.0}$ $10^{7.05}$
Tc 341	1:2			27 30	7 42	2 1	3.5×10^6 3.1×10^6	$10^{6.95}$
F 13	1:20	25 26	13 14	3 7	2 4		3.9×10^7 3.3×10^7	$10^{8.3}$ $10^{8.0}$
R 29	1:100	40 18	15 5	5 0	0 0		7.6×10^7	$10^{8.15}$.

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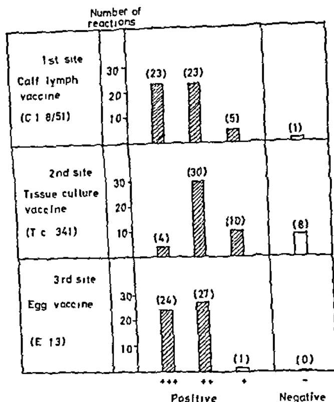


Fig 2

Comparison of the distributions of reaction types obtained after late revaccination with undiluted smallpox vaccines of different origin Each of 52 persons vaccinated at *three sites* with calf lymph egg vaccine and tissue culture vaccine respectively For criteria used to grade reactions see the text

be correlated to vaccine potency Each of 85 men were inoculated with three dilutions (1 4 1 32 and 1 256) of vaccine C1 8 51 At reading 7 days after vaccination each reaction was graded as follows

+++ pock 10 mm in diameter or more either surrounded by a large erythema or resembling a primary reaction
++ pock size 6–9 mm erythema of average size
\+ pock 5 mm or less
— transient vesiculation papule formation or absence of reaction

The distributions of these graded responses for the different dilutions are plotted in Fig 1

The diagrams indicate that variation in reaction types" within a population with a defined homogenous vaccination history is determined to a large extent by the vaccine potency

With the same criteria as used above local reactions following late revaccination with undiluted *vaccines of different origin* were com

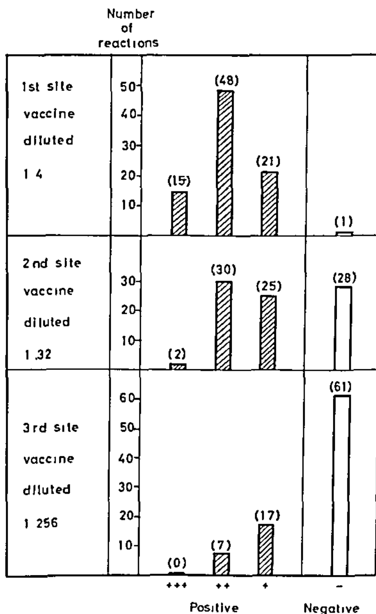
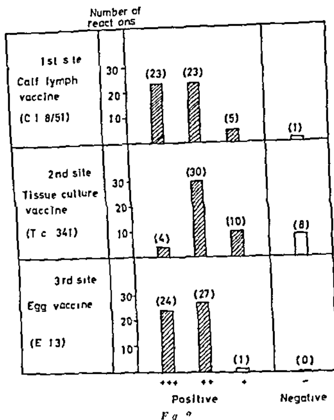


Fig 1

Frequency distributions of reaction types in 85 human adults each revaccinated with 3 serial dilutions of a smallpox vaccine (late revaccinations)
For criteria used to grade reactions see the text

Consequently, early vesicle responses of the mentioned type were classified as "immediate reactions and considered as negative results in this study

b *Quantitative estimation of individual responses to different vaccine dilutions* Although no serious consideration will be paid to graded responses in the sequel, a couple of experiments were made to estimate roughly how the size of local reactions after late revaccination could



F g 9

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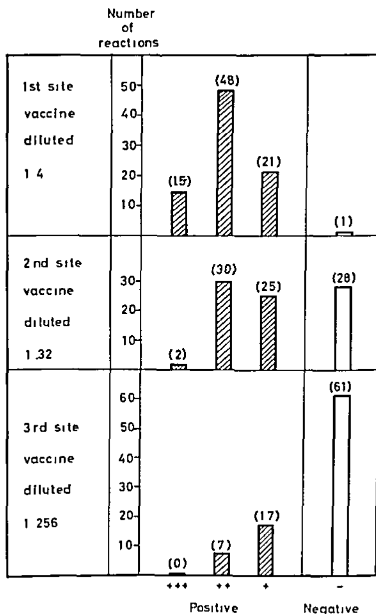


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reactions in the two subgroups, respectively, the difference not being significant The test was repeated on another group of 76 individuals, *group B, using two dilutions* 1 8 $\log_{10}$ apart (64×) in a somewhat different arrangement The result of this test is summarized in the lower part of Table 2 Again, there is no significant indication that, under the test conditions applied, a strong vaccine might influence the quantal response *to a weak vaccine inoculated simultaneously in the* same individual

3 *Results of the Main Vaccination Trial*

The experiments to be decribed were performed during three separate vaccination periods in 1959 The vaccinees consisted of 917 conscripts As to vaccination history the inoculations were primary in 79 persons, late revaccinations in 810 and early revaccinations in 28 The grouping of the material as regards combination of vaccine dilutions, previous vaccination history and differences in reading times is summarized in

TABLE 3 a b and c

Schedule of Arrangements in the Main Vaccination Trial Each Individual was Inoculated Simultaneously at Three Sites with the Set of Vaccine Dilutions Indicated in the First Column The Tables Give Numbers of Individuals According to Vaccination History Combination of Vaccines Applied and Times at which Results were Recorded

3 a) *Calf lymph vaccine C I 8 51*

Set of three vaccine dilutions given to each individual	Number of individuals grouped with respect to vaccination history set of vaccine dilutions applied and reading times					
	Primary vaccinations read after		Late revaccinations* read after		Early revaccinations* read after	
	3 and days	3 and 5 days	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days
1 4 1 32 1 256	10		85	18	3	1
1 8 1 64 1 512	10		100		3	
1 16 1 128 1 1024	7		91	9	1	2
1 1† 1 64 1 64	2		41			
1 64 1 64 1 64	1		35			
	30		279		10	

* Late –

†

pared. Each of 52 individuals was inoculated with calf lymph vaccine (C L 8 51), with egg vaccine (E 13) and with tissue culture vaccine (T c 341). As seen in Fig. 2 the calf lymph and the egg vaccine which had about the same laboratory titer gave essentially similar distributions of the local reaction sizes. With the tissue culture vaccine which was about 10 times weaker the distribution obtained is such as could well be expected in view of the information contained in Fig. 1. From this test there was accordingly no indication that the method of producing the vaccine could *per se* determine the strength of the local reaction.

TABLE 2

Test on Possible Interaction between Different Vaccine Dilutions within Individuals in Late Revaccinations

Test group	Number of persons vaccinated	Vaccine dilutions inoculated into each individual	Frequencies of positive reactions after days		Per cent positive corresponding reactions
A 1	55	10^{-1}	53/55		
		10^{-2}	16/55		
		$10^{-2.5}$	10/55		18.2
A 2	33	$10^{-2.5}$	3/33	17/99	17.2
		$10^{-2.5}$	6/33		
		$10^{-2.5}$	8/33		
B 1	41	10^{0}	38/41		
		$10^{-1.8}$	21/41	48/82	58.5
		$10^{-1.8}$	27/41		
B 2	35	$10^{-1.8}$	19/35	57/105	54.3
		$10^{-1.8}$	18/35		
		$10^{-1.8}$	20/35		

Group A 1 and A 2 Difference not significant
Group B 1 and B 2 Difference not significant

c. *Test for possible interaction between different vaccine dilutions inoculated in the same person.* In order to gain more information from each individual vaccinated in the main trial it was planned that each person should be inoculated with three different vaccine dilutions. As a consequence it was necessary to test whether a strong inoculum would influence the take probability of a weak inoculum applied to the same individual. Two independent tests were performed with 2 vaccine dilution series on two groups (A and B) of conscripts vaccinated more than ten years previously. In the first test on group A consisting of 88 individuals each of the 55 men was inoculated with the vaccine dilutions 10^{-1}, 10^{-2} and $10^{-2.5}$. In the remaining 33 individuals only the weakest dilution ($10^{-2.5}$) was used, being inoculated at all three vaccination sites. The take frequency for dilution $10^{-2.5}$ in the two subgroups was compared at readings 7 days after inoculation. As shown in the upper part of Table 2 there were about 18 and 17 per cent positive

reactions in the two subgroups, respectively, the difference not being significant The test was repeated on another group of 76 individuals, group B, using two dilutions 1 8 $\log_{10}$ apart (64×) in a somewhat different arrangement The result of this test is summarized in the lower part of Table 2 Again, there is no significant indication that, under the test conditions applied, a strong vaccine might influence the quantal response to a weak vaccine inoculated simultaneously in the same individual

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	Primary vac[cinations] read a[t]					
	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days
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	30		279		10	

* Late revaccination > 10 years after last vaccination
Early revaccination 1 3 years after last vaccination
† Reactions with this vaccine concentration not included in the following analysis.

3 b) *Egg vaccine E 13 and tissue culture vaccine T.c. 341*

Set of three vaccine dilutions given to each individual	Number of individuals grouped with respect to vaccination history, set of vaccine dilutions applied and reading times					
	Primary vaccinations read after		Late revaccinations* read after		Early revaccinations* read after	
	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days
E 13 dil. 1:3.16 E 13 dil. 1:31.6 T.c. 341 dil. 1:31.6	9	1	89	11	4	
T.c. 341 dil. 1:3.16 E 13 dil. 1:100 T.c. 341 dil. 1:100	9	4	68	28	6	3
E 13 dil. 1:10 T.c. 341 dil. 1:10 E 13 dil. 1:316	8	1	76	16	2	2
	32		288		17	

* See note of Table 3 a)

3 c) *Egg vaccine E 29*

Set of three vaccine dilutions given to each individual	Number of individuals grouped with respect to vaccination history and set of vaccine dilutions applied		
	All individuals read after 3 and 6 days		
	Primary vaccinations	Late revaccinations*	Early revaccinations*
1:31.6 1:31.6 1:31.6	2	43	
1:100 1:100 1:100	3	47	
1:316 1:316 1:316	4	53	1
1:316 1:1000 1:3160	8		
	17	143	1

* See note of Table 3 a)

Table 3 a, b and c. In most persons different vaccine dilutions were used with a maximal difference of 64 times between the extreme potencies. The experiment outlined in Table 3 c, with three identical inoculations per individual, was performed in order to obtain an additional check of possible interference between vaccine dilutions in the same person. It was further hoped that this arrangement might provide in-

TABLE 4 a and b

Summary of Quantal Responses in Adults of three Immunity Categories after Vaccination with Serial Dilutions of Smallpox Vaccines
Figures Refer to Vaccination Sites

4 a) *Calf lymph vaccine CI 8 51*

Vaccine dilution	Primary vaccination a n* read after 3 days	7 days	Late revaccination a n	Per cent positive	Early revacc a n
1 4	10/10 →	10/10	101/103	98	3 4
1 8	10 10 →	10 10	89/100	89	3 3
1 16	7/7 →	7/7	92/100	92	1/3
1 32	6 10 →	10/10	73/103	71	1/4
1 64	5 17 →	16/17	156/287	54 4	2 3
1 128	6 7 →	7/7	30 100	30	0/3
1 256	2/10 →	9 10	29/103	28	1/4
1 512	1 10 →	5/10	6/100	6	0 3
1 1024	1/7 →	4 7	3/100	3	0 3

4 b) *Tissue culture vaccine and egg vaccines*

	Vaccine dilution	Primary vaccinations a n read after 3 days	5-7 days	Late revaccinations a n	Per cent positive	Early revacc a n
Tissue culture vaccine (Tc 341)	1 3 16	10/13 →	13 13	82/96	85	3 9
	1 10	8/9 →	9 9	49/92	53	2 4
	1 31 6	4/10 →	9 10	21/100	21	2 4
	1 100	3 13 →	4 13	6/96	6	0 9
Chick embryo vaccine (E 13)	1 3 16	10/10 →	10 10	96/100	96	2/4
	1 10	9 9 →	9 9	76/92	83	2 4
	1 31 6	8/10 →	9 10	58/100	58	1/4
	1 100	5 13 →	8 13	29 96	30	2/9
	1 316	2 9 →	3/9	13/92	14	0/4
Chick embryo vaccine (E 29)	1 31 6	6 6 →	6 6	97/129	75	
	1 100	8 9 →	9 9	44/141	31	
	1 316	6 20 →	18 20	17/159	11	0 3
	1 1000	0 8 →	1 8			
	1 3160	0/8 →	0 8			

* a n = Number of positive reactions through number of vaccination sites
Late revaccination = Vaccination more than 10 years after last vaccination
Early revaccination = Vaccination 1 3 years after last vaccination

formation about take variation "within" and "between" individuals respectively

To begin with, single reactions will be considered as independent both biologically and statistically, the data for each vaccine dilution being pooled to arrive at vaccination site take frequencies These quantal responses are shown in bulk in Table 4 a and b From the tables it can be seen that the late revaccination data represent about 100 obser

3 b) *Egg vaccine E 13 and tissue culture vaccine Tc 341*

Set of three vaccine dilutions given to each individual	Number of individuals grouped with respect to vaccination history, set of vaccine dilutions applied and reading times					
	Primary vaccinations read after		Late revaccinations* read after		Early revaccinations* read after	
	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days	3 and 7 days	3 and 5 days
E 13 dil 1:3.16 E 13 dil 1:31.6 Tc 341 dil 1:31.6	9	1	89	11	4	
Tc 341 dil 1:3.16 E 13 dil 1:100 Tc 341 dil 1:100	9	4	68	28	6	3
E 13 dil 1:10 Tc 341 dil 1:10 E 13 dil 1:316	8	1	76	16	2	2
	32		288		17	

* See note of Table 3 a)

3 c) *Egg vaccine F 29*

Set of three vaccine dilutions given to each individual	Number of individuals grouped with respect to vaccination history and set of vaccine dilutions applied		
	All individuals read after 3 and 6 days		
	Primary vaccinations	Late revaccinations*	Early revaccinations*
1:31.6 1:31.6 1:31.6	2	43	
1:100 1:100 1:100	3	47	
1:316 1:316 1:316	4	53	1
1:316 1:1000 1:3160	8		
	17	143	1

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Table 3 a, b and c. In most persons different vaccine dilutions were used with a maximal difference of 64 times between the extreme potencies. The experiment outlined in Table 3 c, with three identical inoculations per individual, was performed in order to obtain an additional check of possible interference between vaccine dilutions in the same person. It was further hoped that this arrangement might provide in-

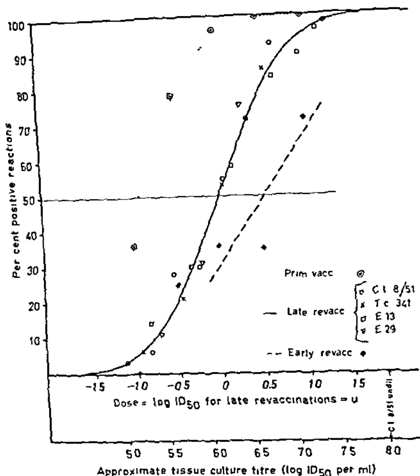


Fig 3

Dose response curves comparing "take frequencies in primary vaccinations late revaccinations (10 years after last vaccination) and in early revaccinations (1-3 years after last vaccination) Pooled data from all four vaccines (Note Early revaccination line only roughly fitted)

and pooled primary vaccinations may well be fitted to normal sigmoid curves when plotted against log vaccine dilution or other logarithmic dose scales

(b) Concerning at least the late revaccinations the four different vaccines form equal curves with no significant deviation from parallelism The dose distances between the curves correspond approximately to differences in laboratory titer

(c) *In the pooled material, graphically illustrated in Fig 3, marked differences are noted between take rates obtained with comparable vaccine potencies in primary vaccinations and in late revaccinations (>10 years after last vaccination)* For equal take

vations per dilution and that essentially the whole range from high to low take frequency is covered as the vaccine potency decreases. It is also evident from the table that the success rate with corresponding dilutions is higher in primary vaccinations than in revaccinations.

If the take frequencies (percentages) obtained in the late revaccinations are plotted against the logarithms of vaccine dilutions, approximately parallel point sequences will result, from which the 50 per cent "dose" for each vaccine can be estimated with good accuracy. If this "50 per cent infective dose for late revaccinations" (abbreviated ID_{50} rev.) is used as a relative titer unit to express the potencies of all vaccine dilutions, a common dose scale is obtained. In this way all data for the four vaccines will be pooled, which is desirable for comparisons between the three categories of vaccinees, since the take frequencies for primary vaccinations and early revaccinations are based on small numbers of observations.

The prerequisites for such a pooling and the way in which it is carried out have been outlined in a separate paper dealing with probit analysis of the data from this study (13).

The different frequency series obtained after pooling and grouping are shown in Table 5.

TABLE 5

Dose-Response Relationships in Primary Vaccinations and Early Revaccinations Relative Vaccine Potencies Grouped in Half $\log_{10}$ Classes and Corresponding Take Frequencies Pooled within Classes

Classes of potency in terms of number of ID_{50} for late revacc.		Primary vaccinations a/n* read after			Early revaccinations a/n	Estim. take rate in late revacc. Per cent	Estim. tissue culture titer† log ID_{50}/ml
Range	Class mid points in log units	3 days	5-7 days	Per cent take			
$10^{1.75}$ – $10^{1.25}$	1.5	10/10 →	10/10		3/4	99.2	7.64
$10^{1.25}$ – $10^{0.75}$	1.0	20/20 →	20/20	100	5/7	94.5	7.14
$10^{0.75}$ – $10^{0.25}$	0.5	42/45 →	45/45	100	7/20	78.8	6.64
$10^{0.25}$ – $10^{-0.25}$	0.0	35/52 →	50/52	96	5/14	50	6.14
$10^{-0.25}$ – $10^{-0.75}$	−0.5	17/53 →	44/53	83	5/20	21.2	5.64
$10^{-0.75}$ – $10^{-1.25}$	−1.0	7/47 →	17/47	36	0/19	5.5	5.14
$10^{-1.25}$ – $10^{-1.75}$	−1.5	(0/8 →	0/8)			0.8	

* a/n = Number of positive reaction through number of vaccination sites.
† Based on the titer of the undiluted vaccine C1 8/51 = $10^{8.0}$.

Estimated take per cent in late revaccinations and estimated tissue culture titers are given for comparison.

The following brief statements, supported by the statistical analysis quoted, may be made with respect to distribution of take frequencies, comparison between vaccines and comparison between the different groups of vaccinees.

(a) Take frequencies from late revaccinations with single vaccines

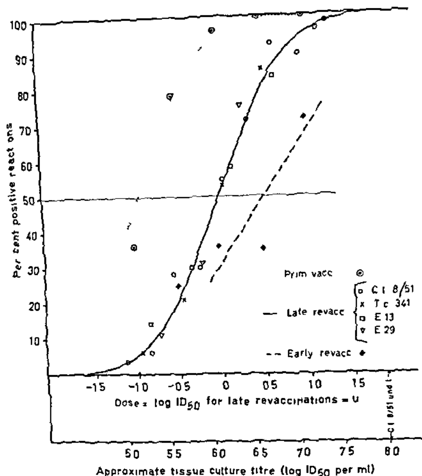


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Dose response curves comparing "take" frequencies in primary vaccinations late revaccinations (10 years after last vaccination) and in early revaccinations (1 3 years after last vaccination) Pooled data from all four vaccines (Note Early revaccination line only roughly fitted)

and pooled primary vaccinations may well be fitted to normal sigmoid curves when plotted against log vaccine dilution or other logarithmic dose scales

(b) Concerning at least the late revaccinations the four different vaccines form equal curves with no significant deviation from *parallelism* The dose distances between the curves correspond approximately to differences in laboratory titer

(c) In the pooled material, graphically illustrated in Fig 3, marked *differences* are noted between take rates obtained with comparable vaccine *potencies in* primary vaccinations and in late revaccinations (>10 years after last vaccination) For equal take

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Classes of potency in terms of number of ID_{50} for late revacc.		Primary vaccinations a/n* read after			Early revaccinations a/n	Estim. take rate in late revacc. Per cent	Estim. tissue culture titer† log ID_{50}/ml
Range	Class mid points in log units	3 days	5-7 days	Per cent take			
$10^{1.75}-10^{1.25}$	1.5	10/10 →	10/10		3/4	99.2	7.64
$10^{1.25}-10^{0.75}$	1.0	20/20 →	20/20	100	5/7	94.5	7.14
$10^{0.75}-10^{0.25}$	0.5	42/45 →	45/45	100	7/20	78.8	6.64
$10^{0.25}-10^{-0.25}$	0.0	35/52 →	50/52	96	5/14	50	6.14
$10^{-0.25}-10^{-0.75}$	−0.5	17/53 →	44/53	83	5/20	21.2	5.64
$10^{-0.75}-10^{-1.25}$	−1.0	7/47 →	17/47	36	0/19	5.5	5.14
$10^{-1.25}-10^{-1.75}$	−1.5	(0/8 →	0/8)			0.8	

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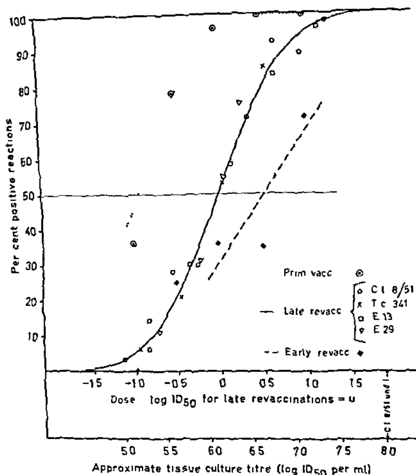


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TABLE 6

Comparison of the Estimated Laboratory Titers of the Four Smallpox Vaccine at the Vaccine Concentrations which Give Similar Quantal Response (i.e. 50 per Cent Take) in Late Revaccinations

Vaccine	"Human titers" of the undiluted vaccines = log ID_{50} in late revacc.	Estimated laboratory titer of vaccine dilution giving 50% take in late revacc.	
		Chick embryo Pfu/ml	Tissue culture log ID_{50}/ml
C.l. 8/51	1.86	5.92	6.12
T.c. 341	1.05	5.47	5.90
E 13	1.66	5.90	6.39
E 29	1.78	6.10	6.37

rates the latter category needed about 9 times stronger vaccine than the former (with a 95 per cent confidence interval from 6 to 14 times). The two corresponding curves are approximately parallel.

The results from the recently vaccinated persons (*i.e.* 1–3 years after the last vaccination) are too scarce and scattered for any valid analysis. The frequencies have, however, been plotted in Fig. 3. It seems that this point sequence is not parallel to the other curves, but that the slope is flatter. If this difference in slope is significant it ought to be interpreted as a greater variation between persons of that population. However, this group was selected by personal questioning as to the positive outcome of the last vaccination, and some answers may have been incorrect leading to heterogeneity of the sampled group with respect to immunity, as well as to overestimation of immunity. On this assumption any comparison with, for instance, the primary data will give minimum estimates.—Measurement from the curves in Fig. 3 shows that the difference of vaccine potencies required in primary vaccinations and early revaccinations on the 50 per cent takes leevel is about 1.5 $\log_{10}$ units (= 32 times). On higher take levels, however, the difference seems to be even larger.

4. *Relation between Laboratory Titers and Vaccination Results*

In practice the anticipation of the take efficiency of a vaccine can be bases only on laboratory titer values. The reliability of such an inference is illustrated in Table 6 showing laboratory titers of the four vaccines in relation to "human titers". The vaccine potency required for obtaining 50 per cent of takes in late revaccinations was estimated by egg titers rangang from $10^{5.5}$ to $10^{6.1}$ pfu/ml and by tissue culture titers from $10^{5.9}$ to $10^{6.4}$ ID_{50}/ml. The variation of the separate titer estimates is larger than the combined errors of the corresponding human ID_{50} estimates and of the laboratory titers. Thus the vaccine T.c. 341, for instance, produced a somewhat higher rate of takes than was expected both by egg titers and tissue culture titers. It is reasonable to infer that discrepancies in these comparisons may be partly due to

the effect of handling the vaccines prior to vaccination, or they may be caused by differences in the inoculation procedure at titration and vaccination respectively which could favour or disfavour a vaccine according to its physical state, etc

DISCUSSION

This study of the relative susceptibility of various human populations to vaccinia virus infection has utilized methods essentially similar to the familiar laboratory procedure for testing relative virus sensitivity of different growth media by making 50 per cent end point titrations in samples of each host and comparing the titers In view of common laboratory *in vitro* experiences, and several published animal experiments (e g *Parker* (23), *Tigertt et al* (26)), and even human vaccinations (*Cockburn et al* (4)), the finding of sigmoid dose reponse relationships in the present study was not unexpected in itself However, by combining three variables—i e dose, response and prevaccination immunity—in a single experiment, as was done in this study, some important information was obtained, which is relevant to smallpox vaccine standardization

As in other sigmoid response relationships most weight is carried by the data in the middle of the curve, i e near the level of 50 per cent takes whereas the possibility of distinguishing different sizes of stimuli by response proportions is increasingly poor the more the takes approach 100 or 0 per cent It seems probable that in the past the demands of official regulations with the view of attaining "the highest possible success rate" have been so respected even by experimentators that conscious utilization of the more profitable middle take rate region was not attempted for systematic quantitative and comparative investigation In fact one of the most valuable reports on dose response relations originated from a vaccine stability trial in which one vaccine unexpectedly failed to retain its potency (4)

The validity of the information gained in the present study is intimately connected with the precision by which the variables representing dose, response and immunity status can be defined

The dose variable has been treated in the same manner as in laboratory titrations—i e serial dilutions have been used and separate doses have been considered as not subject to error This seemed preferable to titrated doses as it would *a priori* reduce the variability in the response variable, and also facilitate the regression analysis since the d[illegible]

[illegible] sensitive cells through the inoculation The effective dose level and its variability will thus finally be dependent also upon the inoculation efficiency and uniformity, respectively In this study attempts were made to ensure the minimum technical varia-

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each vaccine as an end point titer, whereby all the dilutions could be transposed to a common dose scale to facilitate pooling This relative potency unit corresponded to $10^{6.1}$ to $^{6.7}$ $TCID_{50}$ in monkey tubes The primary vaccination data (pooled) likewise showed a sigmoid appearance though this point sequence was incomplete in its lower part (see Fig 3) There was no distinct difference in the shape or the slope of the curves for the late revaccinations and the primary vaccinations, the common slope in the dose-probit diagram being estimated at $b =$ 1 60 However the dose distance between the two curves was 0 96 log units corresponding to about 9 fold smaller doses for primary vaccinations The primary curve had its 50 per cent intercept at a titer of about $10^{4.7}$ $TCID_{50}$ in monkey tubes Finally the take frequencies obtained in early revaccinations (1–3 years since the last vaccination), although scarce and irregular, indicate that this category of persons may need 30 50 times larger doses than the primary group, or on high take levels possibly even more

It thus seems justifiable to suggest that among persons attending common smallpox vaccinations, susceptibility in terms of required vaccine potency may have a 100 fold range (cf Fig 3) If one and the same vaccine is to be used for these different populations the recommendation as to suitable titer level cannot be based solely on the ability to give a high primary take rate The data obtained here also indicate that a vaccine titer of $10^{8.0}$ $TCID_{50}$/ml will give a satisfactory take rate in most human populations dealt with in practice On the other hand this is more than 10 times in excess of the titer needed for practically 100 per cent takes in primary vaccination in adults As reported elsewhere (14) there is a marked positive correlation between vaccine potency and the severity of vaccination reactions The disadvantage of early and large reactions may be accepted in cases where a rapid vaccination effect is wanted, because immunity is then probably established more quickly But if estimated after the passing of the acute post-vaccinal period, the immunity seems to be mainly unrelated to the size (or the number) of reactions at least when measured as the level of neutralizing antibody (cf *Cross* (6) and *Downie et al* (8))

The above finding could apparently lead to consideration of the use of two or more vaccine potencies differentiated according to the expected sensitivity of the individuals to be vaccinated

SUMMARY

Serial dilutions of four smallpox vaccines (one calf lymph one vaccine from suspended bovine embryonic skin, and two egg vaccines) were used in vaccination of male human adults to investigate the connection between local response and vaccine potency, vaccine origin and in particular vaccination history All vaccines gave parallel take frequency curves of the normal sigmoid type when plotted against a logarithmic

tion by having all inoculations done by the same vaccinator and completed within a short period of time

Dilution in itself may produce a dose error since the protective effect of host material upon virus in the vaccine will also be reduced, implying that much diluted vaccine might be more apt to deteriorate This hazard called for careful freeze storage of vaccines up to the very moment of vaccination

The response variable has consisted of take frequencies obtained with the serial vaccine dilutions Distinction thus concerned only "take or "no take", *i e* recognition of morphological changes considered to indicate a process of viral multiplication This contains no difficulty in the primary vaccination, but in revaccination the local reaction is influenced by the booster response including a delayed hypersensitivity ("allergic") component (27, 1) This will cause an allergic skin reaction to the virus antigen, generally appearing before signs of viral growth are visable, and initially independent of this process, but later causing morphological modifications of the cutaneous response to the viral growth through continued allergic reaction to produced viral antigen The period of virus multiplication is also shortened If this period is sufficiently short, difficulties may arise in interpreting the response The risk of the initial allergic skin reaction being misinterpreted as a sign of virus growth in the absence of a take is now generally recognized and has been studied by several authors (*e g Broom* (3), *Benenson* (2), *Cross* (5)) Vesiculation alone has been found not entirely reliable, but should be taken together with time relationships to distiguish false and true "takes" the allergic reaction comes early and is of short duration and as a rule regresses after 4 days, whereas signs of virus growth are progressive or not yet visible by that time Exceptions from this, *i e* when the allergic reaction is large and late (cf *Benenson* (2)), or the incubation time is longer than 5 days, are rare and have not been considered as a serious source of error in this study

Application of 3 different doses with up to a 64-fold range in the same individual might be suspected to cause interaction between vaccination sites, particularly a suppressing effect of a higher upon a lower dose Such phenomena on either immunological (cf *Rosenthal & Libby* (25)) or non immunological basis (cf *Dalldorf et al* (7), *Isaacs et al* (19)) have been observed in several other connections In testing experimental conditions in the present study no such interaction could be shown between doses spaced by 1 8 decadic logarithms

The question of statistical independence between sites within individuals has been discussed in a separate communication (13)

In the late revaccinations (more than 10 years after the previous vaccination) the take proportions given by the dilution series of the four vaccines could be fitted to parallel normal sigmoid curves, which could be rectified by probit transformation The similarity of the curves justified the use of the estimated 50 per cent take dose (dilution) of

each vaccine as an end-point titer, whereby all the dilutions could be transposed to a common dose scale to facilitate pooling. This relative potency unit corresponded to $10^{6.1}$ to $^{6.2}$ $TCID_{50}$ in monkey tubes. The primary vaccination data (pooled) likewise showed a sigmoid appearance, though this point sequence was incomplete in its lower part (see Fig. 3). There was no distinct difference in the shape or the slope of the curves for the late revaccinations and the primary vaccinations, the common slope in the dose-probit diagram being estimated at $b = 1.60$. However, the dose distance between the two curves was 0.96 log units, corresponding to about 9-fold smaller doses for primary vaccinations. The primary curve had its 50 per cent intercept at a titer of about 10 [illegible] $TCID_{50}$ in monkey tubes. Finally, the take frequencies obtained in early revaccinations (1–3 years since the last vaccination), although scarce and irregular, indicate that this category of persons may need 30–50 times larger doses than the primary group, or on high take levels possibly even more.

It thus seems justifiable to suggest that among persons attending common smallpox vaccinations, susceptibility in terms of required vaccine potency may have a 100 fold range (cf. Fig. 3). If one and the same vaccine is to be used for these different populations the recommendation as to suitable titer level cannot be based solely on the ability to give a high primary take rate. The data obtained here also indicate that a vaccine titer of $10^{8.0}$ $TCID_{50}$/ml will give a satisfactory take rate in most human populations dealt with in practice. On the other hand this is more than 10 times in excess of the titer needed for practically 100 per cent takes in primary vaccination in adults. As reported elsewhere (14) there is a marked positive correlation between vaccine potency and the severity of vaccination reactions. The disadvantage of early and large reactions may be accepted in cases where a rapid vaccination effect is wanted, because immunity is then probably established more quickly. But if estimated after the passing of the acute post vaccinal period, the immunity seems to be mainly unrelated to the size (or the number) of reactions, at least when measured as the level of neutralizing antibody (cf. *Cross* (6) and *Downie et al.* (8)).

The above finding could apparently lead to consideration of the use of two or more vaccine potencies, differentiated according to the expected sensitivity of the individuals to be vaccinated.

SUMMARY

Serial dilutions of four smallpox vaccines (one calf lymph, one vaccine from suspended bovine embryonic skin, and two egg vaccines) were used in vaccination of male human adults to investigate the connection between local response and vaccine potency, vaccine origin and, in particular, vaccination history. All vaccines gave parallel take frequency curves of the normal sigmoid type when plotted against a logarithmic

tion by having all inoculations done by the same vaccinator and completed within a short period of time

Dilution in itself may produce a dose error since the protective effect of host material upon virus in the vaccine will also be reduced, implying that much diluted vaccine might be more apt to deteriorate This hazard called for careful freeze-storage of vaccines up to the very moment of vaccination

The response variable has consisted of take frequencies obtained with the serial vaccine dilutions Distinction thus concerned only "take" or "no take", *i e* recognition of morphological changes considered to indicate a process of viral multiplication This contains no difficulty in the primary vaccination, but in revaccination the local reaction is influenced by the booster response including a delayed hypersensitivity ("allergic") component (27, 1) This will cause an allergic skin reaction to the virus antigen, generally appearing before signs of viral growth are visible, and initially independent of this process, but later causing morphological modifications of the cutaneous response to the viral growth through continued allergic reaction to produced viral antigen The period of virus multiplication is also shortened If this period is sufficiently short, difficulties may arise in interpreting the response The risk of the initial allergic skin reaction being misinterpreted as a sign of virus growth in the absence of a take is now generally recognized and has been studied by several authors (*e g Broom* (3), *Benenson* (2), *Cross* (5)) Vesiculation alone has been found not entirely reliable, but should be taken together with time relationships to distinguish false and true "takes" the allergic reaction comes early and is of short duration and as a rule regresses after 4 days, whereas signs of virus growth are progressive or not yet visible by that time Exceptions from this, *i e* when the allergic reaction is large and late (cf *Benenson* (2)), or the incubation time is longer than 5 days, are rare and have not been considered as a serious source of error in this study

Application of 3 different doses with up to a 64-fold range in the same individual might be suspected to cause interaction between vaccination sites, particularly a suppressing effect of a higher upon a lower dose Such phenomena on either immunological (cf *Rosenthal & Libby* (25)) or non immunological basis (cf *Dalldorf et al* (7), *Isaacs et al* (19)) have been observed in several other connections In testing experimental conditions in the present study no such interaction could be shown between doses spaced by 1 8 decadic logarithms

The question of statistical independence between sites within individuals has been discussed in a separate communication (13)

In the late revaccinations (more than *10 years after the previous* vaccination) the take proportions given by the dilution series of the four vaccines could be fitted to parallel normal sigmoid curves, which could be rectified by probit transformation The similarity of the curves justified the use of the estimated 50 per cent take dose (dilution) of

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dosage scale This and crude estimations of the size of reactions indicated that vaccination response was similarly and exclusively related to the potency of the vaccine irrespective of the methods used to produce it

In comparing take rates in three different immunity categories approximately parallel response curves were again obtained In primary vaccinations 50 per cent takes was given by a potency of $10^{\,2}$ $TCID_{50}$/ml (monkey kidney tubes), whereas the corresponding dose needed in late revaccinations (> 10 years since last vaccination) was $10^{6\,1-6\,[illegible]}$ and in early revaccinations (1–3 years since last vaccination $10^{6\,7\ \ 0}$ $TCID_{50}$ per ml To provide 95 per cent takes about 10 times the mentioned potencies were found necessary, for the early revaccinations probably even more

The wide range of susceptibility levels found has important bearings upon determination of potency requirements It is concluded that a vaccine titer of $10^{8\,0}$ $TCID_{50}$ will suffice for most categories of people but it may be preferable to consider a differentiation of the smallpox vaccine potency

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SMALLPOX VACCINATION STUDIES WITH SERIAL DILUTIONS OF VACCINE

2 *Statistical Evaluation of Quantal Response Data*

By

J Åke Espmark

Received 17 vii 64

In a preceding communication (3) a trial was reported in which vaccinations with serial dilutions of 4 different smallpox vaccines were used to determine the relative susceptibility of unvaccinated and partially immune adult populations

The present paper will deal with the statistical treatment of some data from the mentioned study, including probit analysis of quantal responses Methods recommended in textbooks by *D J Finney* (4) and *G W Snedecor* (8) were utilized

1 *The Question of Mutual Independence of Multiple Vaccination Sites in the Same Individual*

Each individual in the trial was inoculated in three separate sites As a rule different potencies of vaccine, not differing more than 64-fold were employed in the three sites The proportions of takes obtained with the various dilutions of the four vaccines in 3 immunologically defined populations are summarized in Table 1 a and b On the basis of vaccination histories the vaccinations were classified as *primary* vaccinations (no previous vaccination), late revaccinations (last vaccination more than 10 years ago), or early revaccinations (last vaccination 1 3 years previously)

Evidently a simple analysis of these data is not possible unless the responses are independent of each other

Through preliminary tests it could be shown that a potent vaccine did not seem to influence the probability of take with a 64 times weaker vaccine in the same individual (3)

Since three responses were recorded in each person it might be relevant to consider the relative rôle of variation between" and within" individuals Extreme dominance of one of these components over the

I am indebted to Prof *S Gard* and Drs *L Heller* and *S Ek* for advice and valuable discussions

other might affect the general interpretation of variation statistics as calculated below

For this purpose some pertinent inference may be drawn from a model experiment with 3 identical inoculations per individual. Five such groups were available in which a single vaccine dilution was used in each group. All persons had been previously vaccinated not less then ten years ago. The obtained distributions of individuals according to number of takes is seen in Table 2, in which also theoretical binomial distributions have been entered for comparison.

TABLE 1 a and b

Summary of Quantal Responses in Adults of Three Immunity Categories after Vaccination with Serial Dilutions of Smallpox Vaccines
Figures Refer to Vaccination Sites

1 a) *Calf lymph vaccine CL 851*

Vaccine dilution	Primary vaccination a/n	Late revaccination a/n	Late revaccination Per cent positive	Early revacc. a/n
1:4	10/10	101/103	98	3/4
1:8	10/10	89/100	89	3/3
1:16	7/7	92/100	92	1/3
1:32	10/10	73/103	71	1/4
1:64	16/17	156/287	54.4	2/3
1:128	7/7	30/100	30	0/3
1:256	9/10	29/103	28	1/4
1:512	5/10	6/100	6	0/3
1:1024	4/7	3/100	3	0/3

(a/n = Number of positive reactions through number of vaccination sites)
Late revaccination = Vaccination more than 10 years after last vaccination
Early revaccination = Vaccination 1–3 years after last vaccination

1 b) *Tissue culture vaccine and egg vaccines*

	Vaccine dilution	Primary vaccinations a/n	Late revaccinations a/n	Late revaccinations Per cent positive	Early revacc. a/n
Tissue culture vaccine (T.c. 341)	1:3.16	13/13	82/96	85	3/9
	1:10	9/9	49/92	53	2/4
	1:31.6	9/10	21/100	21	2/4
	1:100	4/13	6/96	6	0/9
Chick embryo vaccine (E 13)	1:3.16	10/10	96/100	96	2/4
	1:10	9/9	76/92	83	2/4
	1:31.6	9/10	58/100	58	1/4
	1:100	8/13	29/96	30	2/9
	1:316	3/9	13/92	14	0/4
Chick embryo vaccine (E 29)	1:31.6	6/6	97/129	75	
	1:100	9/9	44/141	31	
	1:316	18/20	17/159	11	0/3
	1:1000	1/8			
	1:3160	0/8			

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	1 31 6	9/10	58/100	58	1/4
	1 100	8/13	29/96	30	2 9
	1 316	3 9	13 92	14	0/4
Chick embryo vaccine (E 29)	1 31 6	6 6	97/129	75	
	1 100	9 9	44/141	31	
	1 316	18 20	17/159	11	0 3
	1 1000	1/8			
	1 3160	0 8			

TABLE 2

Distribution of Numbers of Takes in Groups of Individuals Revaccinated with the Same Smallpox Vaccine Dilution on all 3 Vaccination Sites
All Persons Previously Vaccinated More than 10 Years Ago

	Vaccine dilution applied to all 3 sites of each individual in the respective group									
	(1) C 1 8 51 dil 1 64 (35 persons)		(2) C 1 8 51 dil 1 316 (33 pers)		(3) F 29 dil 1 31 6 (43 persons)		(4) F 29 dil 1 100 (47 persons)		(5) F 29 dil 1 316 (53 persons)	
Total site take frequency	57 105 (54%)		17 99 (17%)		97 129 (75%)		44 141 (31%)		17 159 (11%)	
Number of persons with	Found	Theor *	Found	Theor	Found	Theor	Found	Theor	Found	Theor
No take	6	*12*	22	*19*	1	*1*	17	*15*	40	*38*
1 take	12	*14*	7	*12*	5	*6*	18	*21*	10	*14*
2 takes	6	*6*	2	*2*	19	*18*	10	*10*	2	*1*
3 takes	11	*3*	2	*0*	18	*18*	2	*1*	1	*0*

* Frequencies expected on the assumption of binomial distributions

The proportion of individuals in each group expected to show 0, 1, 2 and 3 takes, respectively, may be predicted from the total site take frequency, $\left(\frac{a}{n}\right)$, using the binomial formula

$$f_v = \binom{3}{v} \left(\frac{a}{n}\right)^v \left(1 - \frac{a}{n}\right)^{3-v}$$

where v is the number of takes in an individual (with range from zero to 3) and f_v is the expected proportion of people with v takes

On the assumption of homogeneity within groups, *i e* equal susceptibility of each inoculation site and each individual, and absence of any interaction between sites in the same individual, the expected proportions of 1, 2, 3 and 0 takes in relation to the overall frequency of takes were thus calculated and entered into the composite diagram in Fig 1 Here, the area below the heavy, solid line represents the sum of the fractions of individuals with 0 or 3 takes, *i e* the expected proportion of uniform responses A lack of homogeneity within a group will in most cases show up as a significant deviation from the expected number of uniform responses Interaction between sites, and more specifically mutual inhibition of responses might cause a reduction in the proportion of individuals with three takes All other aberrations from homogeneity will tend to increase the number of uniform responses

When the distribution of individuals with respect to number of takes is close to a binomial, the variation statistics based on number of sites and corresponding numbers of degrees of freedom may be considered as representative both of "within" of "between" variation On the contrary, if variation in susceptibility is large, more individuals than predicted will have a uniform response (0 or 3 takes) If the extreme

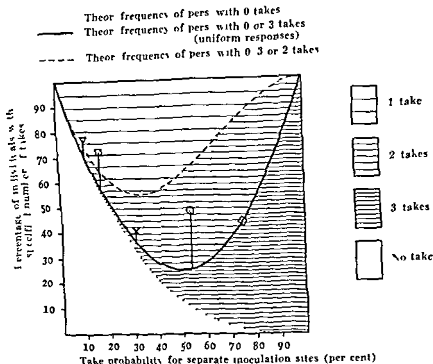


Fig 1

Partitioning of the retical binomial probabilities (expected frequencies in per cent) for 0 1 2 and 3 takes in individuals given three equal inoculations of vaccine with defined site take probability —In the theoretical diagram are entered empirical percentages of unif rm responses (0 or 3 takes) as seen in Table 2 (first series = ○ 2nd = ⊡ 3rd = ◇ 4th = X 5th = ▽)

should occur that all individuals had uniform responses, the adequate number of degrees of freedom should refer to the number of persons rather than to the number of sites since the three sites per individual would offer no more information than one site If, in such a case the variability statistics were computed from total site data only, this would mean over use of information resulting in under estimation of inter personal variation An underestimation on this ground could not exceed $\sqrt{3}$ times expressed in terms of standard deviation

The proportion of uniform responses of the five groups of Table 2 have been plotted into the diagram of Fig 1 The place of the points between the ideal binomial line (unbroken) and the possible extreme (the 100 per cent top line) may visualize the order of magnitude of a possible underestimation of inter personal variation as mentioned above The moderate deviation in at least four of the five series may suggest that the hazard of such errors is small The same conclusion may be indirectly applied to the case with three unequal vaccine doses per

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Number of persons with	Found	Theor *	Found	Theor	Found	Theor	Found	Theor	Found	Theor
No take	6	*12*	22	*19*	1	*1*	17	*15*	40	*38*
1 take	12	*14*	7	*12*	5	*6*	18	*21*	10	*14*
2 takes	6	*6*	2	*2*	19	*18*	10	*10*	2	*1*
3 takes	11	*3*	2	*0*	18	*18*	2	*1*	1	*0*

* Frequencies expected on the assumption of binomial distributions

The proportion of individuals in each group expected to show 0, 1, 2 and 3 takes, respectively, may be predicted from the total site take frequency, $\left(\frac{a}{n}\right)$, using the binomial formula

$$f_v = \binom{3}{v} \left(\frac{a}{n}\right)^v \left(1 - \frac{a}{n}\right)^{3-v}$$

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result in a satisfactory confidence of the take rates It thus seems resonable to use those data for comparisons between vaccines and for the derivation of a relative potency unit to be utilized for pooling the frequencies obtained with the four vaccines

A preliminary impression of the quantal response distribution is obtained by plotting the probit transformed take percentages against the corresponding negative logarithms of vaccine dilutions This is shown in Fig 2 where also straight lines have been drawn to fit the point series Already at this stage some preliminary statements may be attempted which in the following will be tested by computed statistics It seems well conceivable that the point sequences may satisfy straight lines which should be the case if the responses were normally distributed on the dosage scale used The diagram also suggests that these lines might be parallel The point series representing the calf lymph and the two egg vaccines are fairly close together which is in agreement with the laboratory titers (3)

The preliminary probit plot also provides the starting figures for the computational analysis i e the slope of the lines and the dose values corresponding to 50 per cent takes Repeated calculation cycles will then give stepwise improving estimates of the best fitting parallel regression line equations The model equation for regression lines is

$$Y - y = b(x - \bar{x})$$

where Y is the expected probit value derived from the provisional regression line x is the corresponding value for the negative logarithm of vaccine dilution (dose) b is the slope of the regression of probit on "dose y is the weighted average of the corrected experimental probit values (Y) and the weighted average of the dose The weighting procedure includes multiplication of x and y values by n—the number of observations and w—the weighting coefficient obtained from tables (4) and depending on the expected take frequency level — An adequate number of fitting cycles assuming common slopes will give the following probit regression equations for the 4 vaccines

Cl 851	Y	5 0395	=	1 60	(x	1 8399)
T.c 341	Y	4 8314	=	1 60	(x	1 1595)
E 13	Y	5 0903	=	1 60	(x	1 6060)
E 29	Y	4 7007	=	1 60	(x	1 9674)

The dose corresponding to 50 per cent takes ($x_{ID_{50}}$) for each vaccine has been obtained from these equations by substituting the value 5 0 (the probit value for 50 per cent) for Y and has been entered in Table 3 These values may be considered as titers of the vaccines

The slopes (symbolized by b) variances (V) and the sums of squared deviations (D) from the regression lines i e the residual sum of squares (Snw D²) were obtained by usual formulas (4) and were likewise entered in Table 3

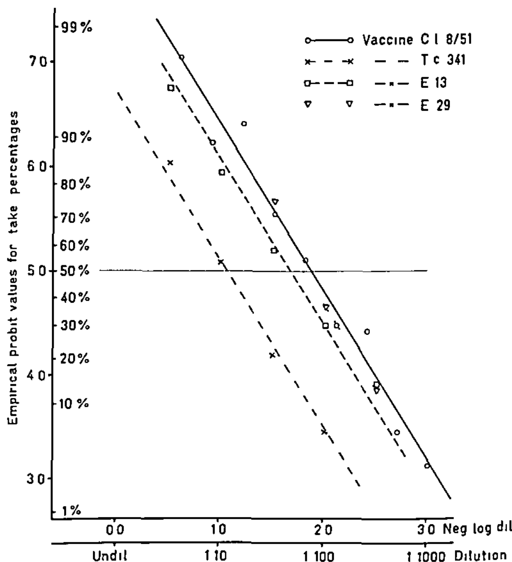


Fig 9

Relationship between dilutions of f r smallpox vaccines and corresponding prol it transformed take percentages in late revaccinations (>10 years after last vaccinat)

individual where similarly a strong domin ince of inter personal varia tion might produce the same sort of error

Moreover the above reasoning is only relevant inasmuch as total variability data are used to estimate variation in susceptibility of per sons but will not considerably influence the essential information of the vaccination trial *i e* the numerical estimates of the relative distances between dose response curves

2 *Probit Analysis*

A *Late revaccination data* From the trial data shown in Table 1 a and b it is seen that the late revaccination category is represented by a relatively large number of observations which would be expected to

result in a satisfactory confidence of the take rates It thus seems resonable to use those data for comparisons between vaccines and for the derivation of a relative potency unit to be utilized for pooling the frequencies obtained with the four vaccines

A preliminary impression of the quantal response distribution is obtained by plotting the probit transformed take percentages against the corresponding negative logarithms of vaccine dilutions This is shown in Fig 2 *where also straight lines have been drawn* to fit the point series Already at this stage some preliminary statements may be attempted which in the following will be tested by computed statistics It seems well conceivable that the point sequences may satisfy straight lines which should be the case if the responses were normally distributed on the dosage scale used The diagram also suggests that these lines might be parallel The point series representing the calf lymph and the two egg vaccines are fairly close together which is in agreement with the laboratory titers (3)

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The dose corresponding to 50 per cent takes ($x_{ID\ 50}$) for each vaccine has been obtained from these equations by substituting the value 5 0 (the probit value for 50 per cent) for Y, and has been entered in Table 3 These values may be considered as titers of the vaccines

The slopes (symbolized by b) variances (V) and the sums of squared deviations (D) from the regression lines *i e* the "residual sum of squares (Snw D) were obtained by usual formulas (4) and were likewise entered in Table 3

TABLE 3 a

Summarized Statistics from the Probit Analysis of Quantal Responses Obtained in Smallpox Vaccinations Data Referring to Late Revaccinations and Separate Vaccines

Vaccine	(1) Number of dilutions	(2) $x_{ID_{50}}$ = neg log of dilution giving 50% take in late revacc	(3) Stand error of $x_{ID_{50}}$	(4) Total number of ID_{50} in undil vaccine	(5) b — slope derived from the common probit analysis	(6) Stand error of b	(7) Sum D = weighted residual sum of squares
C I 8/51	9	1 865	0 036	73	—1 575	0 108	14 748
T c 341	4	1 054	0 055	11	—1 744	0 187	0 411
E 13	5	1 662	0 064	46	—1 394	0 154	0 973
E 29	3	1 780	0 049	60	—1 901	0 215	2 347

TABLE 3 b

Comparative Statistics from Pooled Late Revaccinations and Primary Vaccinations

	(1) Number of dilutions	(2) $u_{ID_{50}}$ log number of 50% late revacc doses needed to give 50% takes in the respective population	(3) Stand error of $u_{ID_{50}}$	(5) b = slope in the u scale	(6) Stand error of b	(7) Sum D — weighted residual sum of squares
Pooled late revaccinations	21	0 000	0 024	1 603	0 075	25 412
Pooled and grouped primary vaccinations	5	—0 965 $\left(=\log \frac{1}{9\ 23}\right)$	0 076*	2 086	0 266*	1 164

* No heterogeneity factor included

Two different residual sums of squares can be calculated to test heterogeneity and parallelism The first is the sum of the residual sums of squares for each separate vaccine, the other (total sum) is derived from the pooled sums of squares and products for all vaccines The difference between the two values may be ascribed to deviation from parallelism

The analysis of heterogeneity and parallelism is summarized in Table 4 Heterogeneity, if present, would be indicated by the total residual sum of squares being significantly larger than the number of degrees of freedom From Table 4 it appears that the total sum of squares exceeds the number of degrees of freedom by a factor 1 588 (heterogeneity factor = H) This may indicate a slight heterogeneity of the quantal response data but will hardly invalidate the assumption of their normal sigmoid distribution Variances obtained in the probit analysis will be corrected by multiplication with the heterogeneity factor

TABLE 4

Analysis of Mean Residual Squares (≈ chi squares) for Testing Heterogeneity and Deviation from Parallelism of the Four Dose Response Series Obtained in Late Revaccinations with 4 Different Smallpox Vaccines

Source of heterogeneity	Degrees of freedom	Residual sum of squares	Mean squares
Parallelism of regressions	3	6 933	2 311
Residual heterogeneity	13	18 479	1 421
Total	16	25 412	(1 588)

$$\text{Variance ratio } F = \frac{2\,311}{1\,421} = 1\,626 \text{ (not significant)}$$

Mean total residual square = 1 588 = heterogeneity factor (H)

The parallelism of the regressions may be tested by the variance ratio (F) of *Fisher*. The ratio of the partitioned mean squares in Table 4 is 1 626 which does not indicate a significant deviation from parallelism

B *Pooling and grouping of vaccine dilution potencies* The analysis of the late revaccination figures provides a basis for pooling the whole material so that the small frequencies for primary vaccination and early revaccinations may be grouped in classes with satisfactory numbers of observations The vaccine potency unit to be used is the amount of virus contained in the vaccine dilution estimated to give 50 per cent *takes in the late revaccination* In the sequel this will be abbreviated ID_{50} rev For several reasons this unit will be preferred to laboratory titers *as far as comparisons within the vaccination trial are concerned* The human titers for the undiluted vaccines may thus be read from Table 3

Vaccine C1 8 51	$10^{1\,86}$ or 73 ID_{50} rev
Vaccine Tc 341	$10^{1\,0}$ or 11 ID_{50} rev
Vaccine E 13	$10^{1\,66}$ or 46 ID_{50} rev
Vaccine E 29	$10^{1\,78}$ or 60 ID_{50} rev

Each of the 2 fold ($= 10^{0\,3}$ fold) or 3 16 fold ($= 10^{0\,5}$ fold) vaccine dilutions may be expressed on similar principles The dilution series of the vaccine C1 8 51 (1 4 1 8 1 1024) for instance will be written as $10^{1\,26}$ $10^{0\,96}$ $10^{-1\,14}$ ID_{50} rev etc

This dose scale unlike that hitherto used (negative logarithms of dilutions) is positive *i e* high dose values indicate high potency etc Relative distances within the scale will be unaffected and slopes will merely change their sign from — to + The dose variable in this scale will be called u (instead of x) The probit regression equations for late revaccination data will be the same for all the vaccines *i e*

$$Y - 5 = 1\,6\,(u - 0) \text{ or } Y = 5 + 1\,6\,u$$

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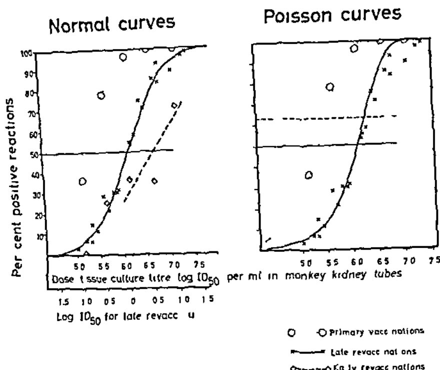


Fig 3

Quantal responses after primary vaccinations and late revaccinations (> 10 years after last vaccination) with varying doses of smallpox vaccine
Left normal curves fitted Right Poisson curves fitted by eye

The pooled data for late revaccinations and primary vaccinations are illustrated in the dose per cent diagram on the left hand side of Fig 3 The dose scale is given both in units of log ID_{50} rev and as tissue culture end point titers

Early revaccinations were too few to yield data of sufficient weight for statistical treatment (see Table 5) However the take proportions have been entered in the left hand diagram of Fig 3 to give a rough suggestion of the susceptibility of this relatively resistant population

DISCUSSION AND SUMMARY

Quantal responses obtained with serial dilutions of four smallpox vaccines in different human populations were submitted to probit analysis

dose response data
was obtained both
ween at least
accinations)
the shape of

It seems convenient for the treatment of primary vaccination data to divide the potency range thus obtained into half-log classes, fitting the middle of the central class to 10^0, *i.e.* one ID_{50} rev.

C. *Primary vaccination data.* The grouping of vaccine potencies in half-log classes and the corresponding pooled take frequencies for primary vaccinations and early revaccinations are presented in Table 5. Only primary data will be considered first. The figures in the first and last class will no be included in the analysis since they carry very little weight. The primary take frequencies of the 3rd–6th classes, now bases on about 50 observations each, are composed of 5, 5, 4 and 5 partial frequencies, respectively, from Table 1. Since there was no evident asymmetry of the partial frequencies within the respective classes there is no suggestion of any considerable bias concerning potency having been introduced by the pooling. For comparison the estimated take percentages for the late revaccinations have been entered in Table 5 as well as the estimated tissue culture ID_{50} titers referring to values for vaccine C.I. 8/51 (titer of the undiluted vaccine $10^{8.0}$ $TCID_{50}$ per ml).

The probit analysis of the primary take data assuming the same slope as above (1.60), will provide an estimate of the potency giving 50 per cent takes as $10^{-0.96}$ ID_{50} rev., *i.e.* about 9-fold lower than for late revaccinations. The standard error of the difference was $10^{0.09}$. Further statistics are entered in the last row of Table 3 b.

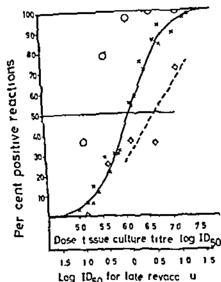
In comparing the slopes of the primary and late revaccination series no significant deviation from parallelism (95 per cent level) could be shown by testing partitioned mean residual squares in the same way as shown in Table 4.

TABLE 5

Dose Response Relationships in Primary Vaccinations and Early Revaccinations. Relative Vaccine Potencies Grouped in Half log_{10} Classes and Corresponding Take Frequencies Pooled Within Classes.
For Comparison Are Given Estimated Take per Cent in Late Revacinations and Estimated Tissue Culture Titers

Classes of potency in terms of number of ID_{50} for late revacc.: Range	Class midpoints in log units u	Primary vaccinations a/n read after 3 days	5- days	Per cent takes	Early revacc. a/n	Estim. take rate in late revacc. per cent	Estim. tissue culture titer* (log ID_{50}/ml)
$10^{1.75}$ – $10^{1.25}$	1.5	10/10 →	10/10		3/4	99.2	7.64
$10^{1.25}$ – $10^{0.75}$	1.0	20/20 →	20/20	100 → 100	5/7	94.5	7.14
$10^{0.75}$ – $10^{0.25}$	0.5	42/45 →	45/45	93 → 100	7/20	78.8	6.64
$10^{0.25}$ – $10^{-0.25}$	0.0	32/52 →	50/52	62 → 96	5/14	50	6.14
$10^{-0.25}$ – $10^{-0.75}$	−0.5	1?/53 →	44/53	32 → 83	5/20	21.2	5.64
$10^{-0.75}$ – $10^{-1.25}$	−1.0	7/47 →	17/47	15 → 36	0/19	5.5	5.14
$10^{-1.25}$ – $10^{-1.75}$	−1.5	(0/8 →	0/8)			0.8	

a/n = Number of positive reaction through number of vaccination sites.
* Based on the titer of the undiluted vaccine C.I. 8/51 = $10^{8.0}$



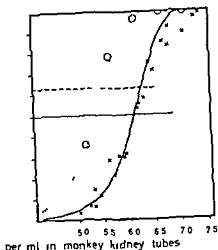


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DISCUSSION AND SUMMARY

Quantal responses obtained with serial dilutions of four smallpox vaccines in different human populations were submitted to probit analysis

The analysis established a satisfactory fit of the dose response data to normal sigmoid curves Approximate parallelism was obtained both in the comparison between the different vaccines and between at least two of the populations (primary vaccinations and late revaccinations)

An alternative distribution will be briefly considered If the shape of

the dose-response relationships were attributable only to frequency distributions of separate infectious units in the inocula, curves of the Poisson type would rather be expected. Such curves have been fitted by eye to the replotted take frequency points as seen on the right in Fig. 3. Apparently the fit is less satisfactory here than with normal curves.

The Poisson distribution does not account for host variation, and in fact this form was found by *Parker* (6) in testing the one-hit infection theory with vaccinia virus in a test system where host variation was minimized. Various factors may affect the shape of the response distribution. With increasing host variation the response curve will tend to become successively flatter than the Poisson curve. Such a distribution may be looked upon as the sum of several parallel Poisson curves, each corresponding to a group of host items with uniform susceptibility. Furthermore, dose response relationships found in practice often fit better to the symmetrical, normal type of curve forming the basis of probit analysis. It may be suggested from Fig. 3 that the late revaccination point sequence is slightly flatter than the inserted Poisson curve, whereas primary data show a better agreement with this form, possibly indicating less variation in susceptibility. This hinted difference of slopes was not, however, large enough to show up in the significance test.

Thorough discussions of quantal responses in relation to the distribution of infectious particles in inocula have been presented by *Gard* (5) and by *Armitage* (1).

The slopes of the regressions of probit upon log dose (about 1.60) are similar in magnitude to those found in primary vaccinations by *Cockburn et al.* (2) and by *Polak et al.* (7).

The practical applications of the results of this analysis are discussed at length in another connection (3).

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THE PRODUCTION OF ANTI-Lp(a)-SERA IN RABBITS

By

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The Lp system of human serum β lipoprotein (*Berg* 1963), can at present only be studied by means of absorbed rabbit immune sera, anti-Lp(a) sera These antisera react in agar gel double diffusion experiments (*Ouchterlony* 1958) only with human sera possessing the genetically determined Lp(a) factor *Berg* (1963) described the production of the first anti-Lp(a)-sera in rabbits The animals were given comparatively large intravenous doses of isolated β-lipoprotein from one single individual The immune sera obtained were submitted to an absorption procedure After the first experiments, *Berg* (1963) discussed the different steps of the immunization procedure, and concluded that it was not possible to state whether any of them might be substituted by alternative techniques The absorption procedure, however, was obviously essential for the production of specific anti-Lp(a) sera Different absorption experiments are described in another paper (*Berg* 1965)

Antisera for routine Lp typing must be specific after absorption in tubes, giving distinct precipitates with all Lp(a+) human sera and no precipitate with Lp(a—) human sera The reaction should preferably develop within 24–48 hours The antisera used should react only with Lp(a+) and not with Lp(a—) human sera over a wide scale of absorption ratios (ratio between antiserum and Lp(a—) human serum) It is then possible to select an absorption ratio for the typing reagent located on the scale, so that the antiserum is specific at several ratios on both sides So far, it has been found that if an antiserum meets the last requirement, it also fulfils all the earlier requirements mentioned The width of the scale of absorption ratios over which the antiserum reacts only with Lp(a+) human sera is therefore an essential expression of the quality of the anti-Lp(a) serum, and will consequently be used in the present paper for comparison of different immune sera

The purpose of the present work is to investigate, by means of immunization experiments, the possible importance of different factors in the production of specific anti-Lp(a)-sera in rabbits The results obtained serve as a guide for the choice of immunization procedure

MATERIALS AND METHODS

Human Sera

A panel of 20 normal human sera (*Berg* 1964) was used. The sera were always used within a few weeks of storage for the absorption experiments and preferably within a few days of obtaining the blood for isolation of β lipoprotein. To obtain comparable conditions, all absorption experiments with Lp(a—) human serum described in this paper, were performed with one single human serum.

Isolation of Human Serum β Lipoprotein

The β-lipoprotein was isolated by chromatography on hydroxylapatite columns as described by *Hjerten* (1959). Solutions of β lipoprotein for immunization were prepared as described by *Berg* (1964), and used within a few days of preparation.

Freund's Adjuvant

Complete Freund's adjuvant (Difco Laboratories, Detroit 1, Mich. Control No 466446) was used.

Rabbits

Rabbits of different races 4–9 months old, weighing at least 3 kg were used for immunization. The different races appeared to be equally satisfactory for the production of anti Lp(a) serum.

Absorption of Immune Sera in Glass Tubes

All immune sera were absorbed in tubes with Lp(a—) human serum (for control absorption experiments with Lp(a+) human serum were also performed).

Undiluted rabbit immune serum and human serum were mixed in different proportions in glass tubes. The tubes were incubated for one hour at 37° C tilted 10–15 times during this period and kept overnight at 4° C. The mixtures were then centrifuged for 10 minutes at 1600 G, the supernatants were pipetted off and tested at once in double diffusion experiments against the panel sera. The supernatants were stored at —25° C for later use.

Rabbit Anti Human Serum Antisera Produced by Proom's Technique

Six of the rabbit anti-human serum antisera previously produced at the Institute of Forensic Medicine were tested. These rabbits had been immunized with two intramuscular injections of pooled human serum treated as described by *Proom* (1943) and one subsequent injection of 1 ml untreated serum intraperitoneally as described by *Hirschfeld* (1960). Each rabbit had received a total of 6 ml human serum.

Glass Slides

Washed and polished 5 × 5 cm glass slides (Menzel Gläser) were used.

Agar

Rheinagar (Behringwerke AG, Marburg, Op. Nr 18 141 142 147 148 150) was used.

Double Diffusion Tests in Agar Gel

The gel diffusion tests were performed on glass slides, the results recorded and photographic registrations made as described by *Berg* (1964, 1965). Each well was filled with 15 μl reagent.

For the intrabasin absorption of immune sera, a technique previously described (*Berg* 1965) was used.

Immunoelectrophoresis

The immune sera were tested as antisera in immunoelectrophoretic experiments performed according to the method of *Grabar* & *Williams* (1953) with the micro modification described by *Scheidegger* (1955).

Staining

The agar slides were stained according to the method of *Uriel* (1960) as described by *Berg* (1964)

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Four different Lp(a+) individuals were used as sources of immunization material for the rabbits injected with antigen from single human donors These 4 donors appeared to be equally satisfactory For the animals immunized with pooled antigen, a pool consisting of equal parts of either isolated β-lipoprotein or whole human serum from the 20 panel donors was used

A total of 53 rabbits were immunized 6 animals died during the immunization period, so that sera from only 47 rabbits remained to be tested In addition, 6 of the rabbit-anti-human-serum antisera previously produced at the Institute of Forensic Medicine were tested

The standard procedure for the intravenous immunization, was that the total amount of immunization material for each rabbit was distributed over 12 injections of equal amounts of the antigen solution The solution was injected into the marginal vein of the rabbit's left ear daily for the first three days of four successive weeks Blood was collected from the marginal vein of the rabbit's right ear 7 days after the last injection and allowed to clot for 2 hours at 37° C, and overnight at 4° C Serum was then pipetted off, centrifuged free of corpuscular elements, and stored at –25° C until used Ordinarily, the sera were tested for the first time the day after the bleeding of the animals

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All the antisera were submitted to an absorption procedure in glass

tubes with several different ratios between rabbit immune serum and Lp(a—) human serum, and tested against Lp(a+) and Lp(a—) human sera, as shown in Table 1 in a previous paper (*Berg* 1963) For control, a portion of each immune serum was also absorbed in tubes in a similar manner with Lp(a+) human serum All the antisera lost all precipitating ability after absorption with a comparatively small amount of Lp(a+) human serum, similar to what is shown in Table 1 in a previous article (*Berg* 1963) The absorption ratios were listed in the order of increasing amount of human serum to a constant amount of immune serum The first absorption ratio with Lp(a+) human serum, where all precipitating ability was removed from the antiserum then corresponded well to the first absorption ratio with Lp(a—) human serum, where the antiserum only precipitated Lp(a+) human sera As these control absorption experiments with Lp(a+) human serum always led to the expected results, they are not described in detail in the present paper and not included in the figures

The results of the absorption of the antisera in tubes with Lp(a—) human serum are presented in diagrams As the immune sera often yield information on various problems, several sera are included in more than one diagram

The Effect of Immunization with Isolated β Lipoprotein Compared with Whole Human Serum

In this section only the rabbits given the amount of β lipoprotein supposed to be closest to that given in 12 ml whole human serum are included

Eight rabbits (Nos 8, 16, 31, 32, 34, 36, 37, 38) were immunized intravenously, each with 12 ml β lipoprotein solution of the standard concentration from single individuals of type Lp(a+) None of the animals died during the immunization period Four of the rabbits (Nos 31, 32 34, 36) produced very useful anti-Lp(a)-sera Of the others, one (No 16) produced a weak anti-Lp(a)-serum, and one (No 37) produced anti Lp(a) antibody, detectable only by the intrabasin absorption technique Two rabbits (Nos 8, 38) did not produce detectable anti-Lp(a) antibody

Eight rabbits were immunized intravenously, each with 12 ml whole human serum from single Lp(a+) donors One rabbit died during the immunization period so that sera from only 7 animals (Nos 45, 51, 52, 53, 54, 92, 95) were tested Five of the sera (Nos 52, 53, 54, 92, 95) could easily be rendered specific by absorption in tubes, whereas the remaining two (Nos 45, 51) contained anti-Lp(a) antibody, detectable only by the intrabasin absorption technique These immune sera contained antibodies against several other serum proteins, particularly the other macroglobulins of human serum

The results of the absorption experiments are summarized in Fig 1

☐ = Absorption ratios where immune serum reacts only with Lp(a+) human sera

☐ = Absorption ratios where immune serum reacts with all human sera

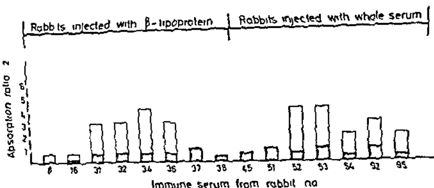


Fig 1

Comparison between sera fr m rabb ts ... nt β lipo-protein solution a ... Lp(a+) ... indiv d ... Each column ... n(s) ... im mune serum Lp(a—) human serum in which the absorption was performed, is here designated "absorption ratio" In the figure the amount of immune serum is kept c nstant ... n Lp(a—) human sera

From these experiments it was evident that it is not necessary to inject the β-lipoprotein in the isolated state to produce anti-Lp(a)-sera, although immunization with the isolated protein prevents the formation of antibodies against other serum proteins

The Effect of Antigen from only One Donor Compared with Pooled Antigen

When it was obvious that whole human serum could be used for immunization, two rabbits (Nos 46, 57) were immunized intravenously, each with 12 ml pooled human serum Both rabbits produced useful anti-Lp(a)-sera (see Fig 2) These antisera are compared with those obtained from the rabbits injected with the same amount of human serum from single Lp(a+) donors in Fig 2

A single rabbit (No 59) which was immunized intravenously with 24 ml of a solution of pooled β-lipoprotein did not produce detectable anti-Lp(a) antibody

The immunization experiments with pooled human serum demon-

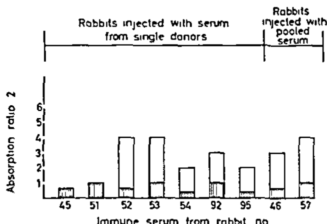


Fig 2

Comparison between sera from rabbits immunized intravenously with 12 ml whole human serum from single Lp(a+) individuals and pooled human serum respectively For further legend, see Fig 1

strated that specific anti-Lp(a) antibody could also be produced by immunizing rabbits with antigen from several individuals, not only from single donors

The Effect of Amount of Antigen Used for Immunization

The following experiments were performed to compare the effect of different antigen doses.

Three rabbits (Nos 1, 2, 3) were each immunized intravenously with a total of 24 ml of isolated β-lipoprotein from one single Lp(a+) individual Eight rabbits (Nos 8, 16, 31, 32, 34, 36, 37, 38) were immunized intravenously, each with 12 ml of a β-lipoprotein solution from single Lp(a+) donors

Two rabbits (Nos 61, 62) were immunized intravenously, each with 24 ml of a 1/10 dilution of a β lipoprotein solution from one single Lp(a+) individual, each animal thus receiving a total dose equivalent to 2 4 ml of the undiluted solution

Seven rabbits (Nos 45, 51, 52, 53, 54, 92, 95) were injected intravenously, each with 12 ml human serum from single Lp(a+) donors

Three rabbits (Nos 87, 88, 89) were immunized intravenously, each with 6 ml human serum from a single Lp(a+) donor

Three rabbits (Nos 55, 56, 60) were each given 12 ml intravenously of a 1/10 dilution of human serum from one Lp(a+) individual, each animal thus receiving a total dose equivalent to 1 2 ml undiluted serum

The results of these experiments are shown in Figs 3 and 4

Several of the rabbits immunized with 12 ml of either β-lipoprotein solution or whole human serum produced very useful anti-Lp(a)-sera All of the three rabbits immunized with 24 ml β-lipoprotein solution produced anti-Lp(a) antibodies, two of the sera (Nos 1, 3) were the best anti-Lp(a)-sera of the whole series, as they were specific over a

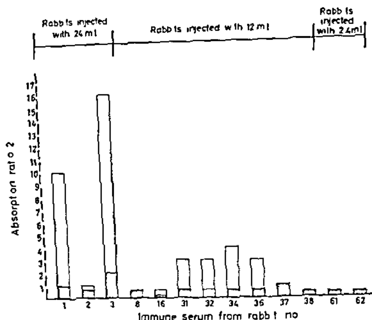


Fig 3

Comparison between sera from rabbits immunized intravenously with different amounts of purified β lipoprotein from single Lp(a+) individuals For further legend see Fig 1

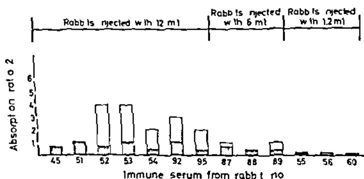


Fig 4

Comparison between sera from rabbits immunized intravenously with different amounts of Lp(a+) human serum from single individuals For further legend see Fig 1

great range of absorption ratios Although subjected to an identical immunization procedure rabbit No 2 produced only a weak anti Lp(a) serum not satisfactory for Lp typing None of the two animals injected with 2 4 ml β lipoprotein solution produced detectable anti Lp(a) anti bodies

Two of the three rabbits (Nos 87 89), given 6 ml whole human se

rum, produced weak anti-Lp(a)-sera None of the animals injected with a total dose equivalent to 1 2 ml human serum produced detectable anti-Lp(a) antibodies Thus, a definite effect of the antigen dose was found Six ml human serum was insufficient to induce the production of antisera satisfactory for Lp typing, and rabbits receiving 1 2 ml whole serum or 2.4 ml β-lipoprotein solution did not produce detectable anti-Lp(a) antibodies at all

The Effect of Route of Immunization

To investigate whether intravenous injection of the rabbits is essential for the results obtained, this route of injection was compared with the intramuscular one In this section only immunization without the use of adjuvants will be dealt with

Seven rabbits (Nos 45, 51, 52, 53, 54, 92, 95) were each immunized with 12 ml whole human serum from single Lp(a+) donors, and 3 rabbits (Nos 87, 88, 89) were each immunized with 6 ml whole human serum intravenously Most of the rabbits of the former group produced usable anti-Lp(a)-sera, whereas none of the latter group provided antisera usable for testing in the Lp system, although antibody could be demonstrated in the sera of two of the three rabbits (Nos 87, 89)

Two rabbits (Nos 91, 93) were immunized intramuscularly, each with a total of 12 ml Lp(a+) whole human serum, distributed over 12 injections Serum from the same human donor had also been used for some of the intravenously injected rabbits (Nos 92, 95)

Three rabbits (Nos 78, 85, 86) were each immunized intramuscularly with 6 ml whole human serum from one Lp(a+) individual which had also been used for immunization of the rabbits that had received 6 ml serum intravenously

None of the intramuscularly injected animals developed anti-Lp(a) antibodies (see Fig 5)

These experiments indicate that of the immunization techniques tested, specific anti-Lp(a) antibodies can only be obtained by intravenous injections

The Effect of Adjuvant and Intramuscular Injection of Antigen

In the first experiments, a total of 6 ml undiluted Lp(a+) β-lipoprotein was given to each of 2 rabbits (Nos 63, 64), a total of 6 ml undiluted human serum each to 2 other rabbits (Nos 65, 66) The antigen solutions were from the donor also used for intravenous injection of 6 ml whole human serum into the earlier described animals The antigen solution was divided into four equal parts, and given as weekly intramuscular injections for 4 weeks, each time mixed with an equal amount of Freund's adjuvant Each rabbit thus received a total of 6 ml of the adjuvant

In a second experiment, three rabbits were immunized intramus-

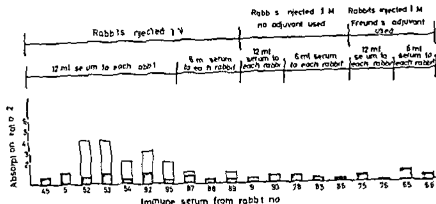


Fig 5

Comparison between sera from rabl its imm m ze l with either 12 or 6 ml Lı (a+) human serum fr m s ngle indiv duals intramuscularly with r without the use of Freund s adjuvant r intravenously with ut adjuvant
For further l gen l see F g 1

cul irly each with 12 ml whole human serum from one single I p(a+) donor mixed with equal parts of Freund s adjuvant The immunization material was distributed over 4 injections as described above Each animal thus received a total of 12 ml of the adjuvant All the rabbits lost weight and appeared cachectic during the last part of the immuni zation period and one died so that sera from only 2 rabbits (Nos 75 76) were tested

The results of testing the sera from rabbits immunized with whole human serum are included in Fig 5 whereas the sera from the rabbits immunized with β lipoprotein solution (Nos 63 64) are not Their gra int of serum No 8) with Freund s ad ed anti I p(a) anti bodies Thus no difference in the response to the I p(a) antigen was observed whether Freund s adjuvant was used or not when the im munization was made by the intramuscular route

Sera from all the rabbits immunized intramuscularly with I p(a+) human serum are included in Fig 5 and compared with those of the animals injected intravenously with the same amounts of human se rum

A difference in the production of anti I ı (a) antibody is observed (see Fig 5) The failure of ... anti I p(a) antibodies tion of the antigen is anti Lı (a) antibody

In addition 6 rabbit anti human serum antisera (Nos 444 450 467

[1] I am indebted to Dr *T Reinskou* Inst tute of Forens c Medi ne Univers ty of Oslo for the pro s on f these sera

468, 486, 501) were also tested[1] As mentioned, the rabbits had been immunized with pooled human serum treated as described by *Proom* (1943) None of these sera contained detectable anti-Lp(a) antibodies

The Development of Anti-Lp(a) Antibody during the Immunization Period

In addition to the tests of the immune sera on completion of the immunization, sera from twelve animals were tested with the intrabasin absorption technique after 1, 2 and 3 weeks of immunization Of these twelve animals, seven produced anti-Lp(a) antibodies when the immunization was completed None of the sera contained anti-Lp(a) antibodies after 1 week of immunization, two after 2 weeks and three after 3 weeks, whereas the remaining four possessed detectable antibodies against the Lp(a) factor only after 4 weeks immunization Of these four, there were two sera in which the anti Lp(a) antibody could only be demonstrated by the intrabasin absorption technique The five rabbits that did not produce detectable anti-Lp(a) antibodies on completion of the immunization period, formed such antibodies in no instance at an earlier stage

The Persistence of Anti-Lp(a) Antibodies in immunized Rabbits

The bleeding of the rabbits about a week after the last injection is based on observations by other authors (*Landsteiner* 1947, *Boyd* 1956), and a systematic investigation of the persistence of antibodies in the rabbits is beyond the scope of this investigation The best rabbit, No 3, was, however, in addition to the routine bleeding, also bled 30 and 40 days after the last injection Rabbit No 1 was, in addition to the routine bleeding also bled 25 days after the last injection Sera from both rabbits had become much weaker in the interval between the routine bleedings and the additional bleedings Serum from rabbit No 3, which was originally specific in the absorption ratios from 2 3 to 2 16, was specific in the absorption ratios from 2 0 4 to 2 1 30 and 40 days after the last injection Serum from rabbit No 1 which was specific in the absorption ratios from 2 2 to 2 10 after the routine bleeding was only specific in the ratios from 2 0 4 to 2 1 25 days after the last injection

The amount of anti-Lp(a) antibodies had thus decreased considerably from the time of the routine bleeding to 25 30 days after the last injection

The Effect of Re Immunization

Two of the intravenously injected rabbits (Nos 2, 3) were re-immunized 2 months, and one (No 52) 6 months after the original immunization The re-immunization consisted of one week's immunization iden

tical to the schedule for one week of the original procedure, and bleeding seven days later.

One of the rabbits (No 2) originally formed an anti-Lp(a) antibody not usable for testing. After re-immunization the quality of the immune serum was essentially unchanged.

After the original immunization two of the rabbits (Nos 3, 52) produced anti Lp(a) antibodies that were excellent for Lp testing (see Figs 3 and 4). After the re-immunization, immune serum from rabbit No 3 was still usable for Lp typing, but was much weaker than before. Serum from rabbit No 52 could not be rendered specific by absorption in tubes after re immunization, but still contained anti-Lp(a) antibodies detectable by the intrabasin absorption technique.

These few experiments seem to indicate that re-immunization is of no advantage for the production of anti Lp(a)-sera.

Immunization of Rabbits with Material from Lp(a—) Individuals

Immunization experiments with antigen solutions from Lp(a—) individuals were performed as a control, and also in order to try to produce antisera revealing a factor complementary to Lp(a).

Seven rabbits were each immunized intravenously with 12 ml isolated β lipoprotein of the standard concentration from Lp(a—) single donors. One of the animals died before the rabbits were bled, and only 6 sera remained to be tested (Nos 4, 5, 6, 7, 17, 33). Three additional rabbits were each immunized intravenously with 12 ml whole human serum from a Lp(a—) individual. Two animals died during the immunization period, so that only one (No 69) remained whose serum could be tested.

The standard procedure for intravenous immunization was applied.

All the immune sera contained antibodies against human β-lipoprotein. No anti Lp(a) antibodies were demonstrable in any of the immune sera obtained. No antibodies against a factor complementary to Lp(a) were demonstrable when these sera were tested similarly to those from rabbits immunized with Lp(a+) material, except for the absorption serum now being of type Lp(a+). Several Lp(a+) sera were used for these absorption experiments and subsequent tests, but so far without success.

The Ag system of *Allison & Blumberg* (1961) also belongs to human β lipoprotein.

The sera from the panel donors ha'
Ag(a) factor by Dr *B S Blumberg* of
Bethesda Maryland. In order to detec
sera from 5 rabbits immunized with material from Lp(a—) Ag(a+) donors were absorbed with a Lp(a—) Ag(a—) human serum, and tested against normal sera. In these experiments, no anti-Ag(a) antibody could be detected in the immune sera.

DISCUSSION

The number of animals necessary to investigate all the problems under study in the present paper completely by far exceeds the number available to the present author. Therefore, at least in respect of some of the problems, the results of the experiments should be interpreted with care. *Berg* (1963) discussed 4 factors which might be essential in the production of anti-Lp(a)-sera, and also claimed that comparatively large amounts of antigen were probably necessary. The importance of one of the factors, the absorption procedure, was already obvious from the first experiments. The present work yields information pertinent to the other factors.

It is not necessary to use isolated β lipoprotein for the immunization to provoke the production of anti-Lp(a) antibodies. The question of "competition of antigens" (see *Boyd* 1956, p. 111) is therefore not an essential one in the production of such antisera. The strongest anti-Lp(a)-sera so far produced (Nos. 1, 3) were obtained by immunization with a solution of isolated β-lipoprotein. These rabbits were, however, given very large amounts of antigen solution so this result probably has more bearing on the question of antigen doses. The rabbits tolerated the large doses of antigen well, whereas the rabbits injected intravenously with 12 ml whole human serum had a tendency to go into a state of anaphylaxis in the 3rd and 4th week of immunization. It is thus possible that the use of a solution of isolated β-lipoprotein is advantageous for immunization, because it is possible to inject large amounts of antigen in this way with less risk of anaphylactic shock.

A disadvantage of using isolated β-lipoprotein is the laborious procedure involved in fractionating human serum.

Good antisera can also be obtained by injecting the rabbits with pooled human serum instead of serum from single Lp(a+) individuals. In the light of what was found regarding the doses of antigen, it is, however, logical to take advantage of the greater amount of Lp(a) antigen obtained by the use of Lp(a+) serum alone, in contrast to a pool containing Lp(a—) serum as well. The lack of response to the injection of Lp(a) antigen in the only surviving animal immunized with pooled β-lipoprotein solution was most probably due to chance.

The correlation between the amount of Lp(a) antigen injected, and the rabbit's response to the immunization, was found whether the animals were injected with whole human serum or isolated β lipoprotein. This correlation may indicate that the Lp(a) factor is a comparatively small part of the β lipoprotein.

It is apparently essential in the production of anti-Lp(a) antibodies that the immunization material is injected intravenously, not intramuscularly. None of the rabbits who received the antigen by intramuscular injection in amounts that provoked the production of anti Lp(a) antibodies on intravenous injection developed detectable antibodies

against the Lp(a) factor whether Freund's adjuvant was added or not. Thus the use of Freund's adjuvant has so far offered no advantage.

The 4 weeks intravenous immunization procedure followed by bleeding of the rabbit one week after the last injection provided good results in our hands and it was beyond the scope of the present work to study in detail the effect of re-immunization, longer immunization periods or the persistence of antibodies in the rabbits after the immunization procedure.

It was not the intention of the experiments described in this paper to investigate the possibility of producing antibodies against a factor complementary to Lp(a), if such a factor exists. The rabbits immunized with antigen solutions from Lp(a—) individuals served above all as controls. These animals never formed antibodies against the Lp(a) factor, neither did they produce antibodies against any hypothetic factor complementary to Lp(a). It is not known why the rabbits should not produce such antibodies, but several explanations are possible. The immunization procedure so far used is perhaps not optimal for this factor. The rabbits may lack the ability to produce antibodies against the factor. In this connection it should be mentioned that *Wheeler, Sawin & Stuart* (1939) have suggested that the ability of rabbits to produce specific anti-M antibodies is genetically determined. Animals of other species should therefore be tried. Experiments utilizing tolerance in newborn animals should also be considered. Such experiments and absorption experiments with the antisera are however difficult to perform satisfactorily as long as there is no possibility of distinguishing between individuals who are homo- and heterozygous for the gene *Lp^a* (see *Berg* 1963).

The results concerning the production of anti-Lp(a) sera should probably be regarded in view of what has previously been done in the field of individual genetic factors of human blood demonstrable by heteroantisera. The discoverers of the MN and P systems used an immunization procedure consisting of one intravenous injection followed by several intraperitoneal injections (*Landsteiner & Levine* 1928). Different immunization procedures have been used since then. Most authorities, however, include several intravenous injections in their procedures (*Race & Sanger* 1954, *Wiener* 1948, *Stratton & Renton* 1958, *Dunsford & Bowley* 1955). Wiener in fact recommends a procedure which in respect of the number of intravenous injections and the length of the immunization procedure is very similar to the one this author has used for production of anti-Lp(a) sera.

The first successful attempt to demonstrate individual antigenic differences in human sera was reported by *Cumley & Irwin* (1943). They immunized their rabbits with 5.4 ml human serum distributed over 9 intravenous injections. *Goldberg & de Gara* (1948) were however unable to reproduce their findings.

The present data indicate that it is essential to inject the antigen

intravenously, not intramuscularly, to obtain anti-Lp(a)-sera The most logical explanation is probably that the antigen from the intramuscular injections does not reach in sufficient amounts or in an *adequately preserved state, a part of the reticuloendothelial system* where anti-Lp(a) antibodies can be produced, to induce the production of such antibodies This raises the interesting question of the site of production of the anti-Lp(a) antibodies If anti-Lp(a) antibodies can be produced in the peripheral lymph nodes, one would expect the rabbits who received large doses of Lp(a+) human serum intramuscularly to be able to produce anti-Lp(a) antibodies The findings may therefore indicate that antibodies against the Lp(a) factor are not produced in the peripheral lymph nodes, but solely in some central part of the reticuloendothelial system The present study gives no indication as to which part this might be It is known from the literature (see *Boyd* 1956, p 75) that the site of antibody formation depends to a considerable extent upon where the antigen is injected

At present, it is not possible to state whether the procedure described for the production of anti-Lp(a)-sera will turn out to be useful in the production of antibodies against as yet unknown serum protein factors as well The detection of a genetic system by means of this technique makes it reasonable to try to find as yet unknown systems in the same manner

SUMMARY AND CONCLUSIONS

1 Immunization experiments have been made in order to study which factors may influence the production of anti-Lp(a) sera in rabbits
2 *Intravenous injections are probably essential for production of* anti-Lp(a)-sera
3 Large amounts of the available solutions of immunization material are essential, 12 ml whole human serum or 12 24 ml of a 0 43 per cent β lipoprotein solution to each animal, distributed over 12 injections, have provided good results
4 Anti-Lp(a)-sera can also be obtained by immunization with whole human serum, not only isolated β lipoprotein
5 The use of antigen from only one donor is not essential To obtain as large an amount as possible of Lp(a) antigen for immunization only serum or isolated β lipoprotein from Lp(a+) individuals should be used
6 Adjuvant *offered no advantage*
7 Because of the different effect of the intravenous and intramuscular route of immunization, the question of the site of anti Lp(a) antibody formation is discussed

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IMMUNOCHEMICAL STUDIES OF ANTI-Lp(a)-SERA

By

KARL BERG

Received 30 vii 64

The Lp system of genetic types within human serum β lipoprotein was discovered by means of antisera produced in rabbits (*Berg* 1963) This system is independent of the Ag system of *Allison & Blumberg* (1961) which also concerns the β-lipoprotein (*Berg* 1964 a) The test method for both systems is the double diffusion technique in agar gel (*Ouchterlony* 1958)

The rabbit immune sera used for typing in the Lp system have been submitted to an absorption procedure after which they possess the anti-Lp(a) specificity, and react only with human sera containing the Lp(a) antigen At present, the Lp system can only be studied by means of these specific heteroantisera The problem of preparing antisera is therefore of central importance Different immunization experiments are described in another paper (*Berg* 1965)

The purpose of the present paper is to identify the specific anti Lp(a) activity of rabbit immune sera as a 7S γ globulin antibody directed against a component of human β-lipoprotein, and to exclude the possibility that the precipitating ability is artificially introduced through the absorption procedure

MATERIALS AND METHODS

Human Sera

Human sera from the standard panel and from other healthy donors (*Berg* 1964 b) were used (see also *Berg* 1965)

Isolation of β Lipoprotein

β Lipoprotein from Lp(a+) and Lp(a—) human sera of the panel was isolated by chromatography on hydroxylapatite columns as described by *Hjerten* (1959)

Standard β Lipoprotein Solution

Different dilutions of a 2 per cent (w/v) β lipoprotein solution prepared in the ultracentrifuge (Op Nr 22263 from Behringwerke AG Marburg) were used[1]

Rabbit Immune Sera

Sera from rabbits immunized as described in another paper (*Berg* 1965) were used Sera from the same rabbits obtained before immunization were used for control

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1 I am indebted to Dr *Heide* and Dr *Storiko* Behringwerke AG Marburg for providing this sample

Sheep Anti Rabbit Serum Antiserum

A sample of antiserum from a sheep immunized with pooled whole rabbit serum was kindly provided by Dr *J Ulstrup* Ullevål Hospital Oslo

Dialysis bags

Cellophane casings from Visking Co Chicago Ill were used

Fractionation of Rabbit γ Globulin

7S and 19S γ globulins of a rabbit antiserum were separated as described by *Greenburg Moore & Nunn* (1963), by precipitation with Na_2SO_4 and chromatography on DEAE cellulose (Cellulose N N Diethylaminoethyl Ether Eastman Organic Chemicals Rochester 3 N Y)

Concentration of Chromatographic Fractions

The fractions were concentrated by dialysis against Polyethylene glycol 20 000 (Hoechst) as described by *Kohn* (1959)

Glass Slides

Washed and polished 5 × 5 cm glass slides (Menzel Glaser) were used

Agar

For the double diffusion tests Rheinagar (Behringwerke AG Marburg Op Nr 18 141 142 147 148 150) and for the immunoelectrophoretic experiments washed Bacto Agar (Difco Laboratories Detroit 1 Mich Control No 452925) were used

Double Diffusion Tests in Agar Gel

The gel diffusion tests were performed on glass slides and the results recorded as previously described (*Berg* 1964 b 1964 c) As a rule the precipitation pattern was fully developed after 2 days

Photographic registrations were made as described by *Berg* (1964 b)

Immunoelectrophoresis

Immunoelectrophoresis was performed as described by *Grabar & Williams* (1953) with the micromodification of *Scheidegger* (1955)

Staining

The agar slides were stained according to the methods of *Uriel* (1960 1961) as previously described (*Berg* 1964 b)

EXPERIMENTS AND RESULTS

Characterization of the Anti Lp(a) Antibody of Rabbit Immune Serum

When the rabbit immune sera were submitted to electrophoresis and allowed to diffuse against human serum or isolated human β lipoprotein a precipitate developed corresponding to the γ globulin region of the rabbit sera (see Berg 1963, Fig 3) This indicates that the antibody of the rabbit immune sera belongs to the γ-globulin

An experiment was performed in which γ globulins from an immune serum containing anti-Lp (a) antibody were purified, and the 7S and 19S fractions were separated by chromatography The procedure of *Greenburg Moore & Nunn* (1963) was followed in detail The crude γ globulin fraction precipitated from 20 ml of the antiserum by addition of Na_2SO_4 was dissolved in 0 85 per cent saline solution and dialysed for several days against several changes of 0 85 per cent saline A sample for testing was secured after the dialysis The protein solution

was then dialysed overnight against the starting buffer, and 5-step chromatography was performed with buffers of increasing molarity and decreasing pH. According to *Greenbury, Moore & Nunn* (1963), the 7S antibody is found in the fractions eluted by the first buffer, and the 19S antibody in the fractions obtained with the last buffer, whereas the fractions eluted by the three intermediate buffers contain much less or no antibody. The main protein-containing fractions eluted by the first buffer were pooled, dialysed against a 0.85 per cent saline solution and concentrated to a total volume of 5 ml. This solution will be referred to as 7S γ-globulin. The main protein-containing fractions eluted by the last buffer were treated in the same manner, and will be referred to as 19S γ-globulin.

These two protein solutions were tested immunoelectrophoretically against a sheep-anti-rabbit-serum-antiserum. This test confirmed that the protein solution obtained by elution with the starting buffer contained 7S γ-globulin, and that obtained with the last buffer 19S γ-globulin. In the solution of 7S γ-globulin, only one line of precipitation could be seen. In the solution of 19S γ-globulin a precipitation arc could be seen in the β-globulin region, in addition to the one in the γ-globulin region. When the 7S and 19S γ-globulin solutions were tested in double diffusion experiments against normal human sera, only the 7S γ-globulin solution formed precipitate with human sera. Attempts were made to detect anti-Lp(a) activity in the 7S γ globulin fraction, by intrabasin absorption with Lp(a—) human serum. 15 μl of human serum was always used, whereas experiments with both 15 and 30 μl of the 7S γ globulin solution were performed. No precipitate developed between the absorbed γ-globulin solution and the panel sera. A sample of the crude γ-globulin solution obtained after addition of Na_2SO_4 to the immune serum was tested in the same manner as the 7S γ-globulin. No precipitate was formed with the panel sera when this protein solution was absorbed with Lp(a—) human serum. It was concluded that the anti-Lp(a) antibody had lost its precipitating ability when the γ-globulin was precipitated by Na_2SO_4.

An experiment was performed in order to find whether the anti-Lp(a) antibody still possessed the ability to inhibit the precipitation of the Lp(a) antigen by anti-Lp(a)-serum. Peripheral wells on agar slides were filled with 15 μl 7S and 19S γ-globulin solutions respectively, and the slides were placed in a moist chamber. When the γ-globulin solutions had diffused into the surrounding gel, 15 μl Lp(a+) human serum was introduced into the same wells, and anti-Lp(a)-serum was placed in the central well. No precipitate developed between anti-Lp(a)-serum and Lp(a+) human serum, when the 7S γ-globulin solution had been filled in the well prior to the human serum, whereas Lp(a+) human serum from the well into which the 19S γ-globulin solution had been introduced formed distinct precipitates with this antiserum. These results are illustrated in Fig. 1.

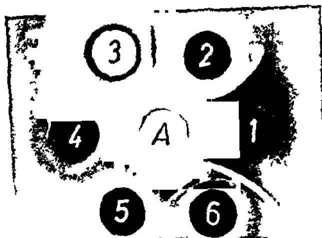


Fig 1

Prevention of precipitate formation in agar gel with anti Lp(a) serum by addition of 7S γ globulin from immunized rabbit to Lp(a+) human serum (see text)

The reagents (15 µl of each) were

A Anti Lp(a) serum
1 Anti β lipoprotein serum from rabbit
2 7S γ globulin from immunized rabbit and Lp(a+) human serum
3 Lp(a+) human serum
4 Lp(a—) human serum
5 0.85 per cent saline solution
6 19S γ globulin from immunized rabbit and Lp(a+) human serum

No precipitate developed between anti Lp(a) serum and the Lp(a+) human serum of well 2 This indicates that the rabbit 7S γ globulin possesses the anti Lp(a) activity

From the inhibiting property of the 7S γ globulin solution, it was concluded that the anti Lp(a) antibody belongs to the 7S γ globulins of the rabbit immune serum

Demonstration of the Anti Lp(a) Activity as a Property Present in Untreated Immune Sera

Sera obtained from the rabbits before immunization were tested by double diffusion in agar against normal human sera No precipitates were formed in these experiments

The sera obtained after immunization were used as antisera against normal human sera in immunoelectrophoretic experiments Each immune serum was tested simultaneously against a Lp(a+) and a Lp(a—) human serum All immune sera gave precipitates with human β lipoprotein Lp(a+) and Lp(a—) human sera giving identical precipitation patterns

All immune sera gave precipitates in double diffusion tests against different dilutions of the standard β lipoprotein solution Most of the

sera reacted with β-lipoprotein solutions of concentrations 0 015 per cent or higher, and some also with 0 007 per cent or higher No correlation was found between the lowest concentration of β-lipoprotein with which the serum gave a visible precipitate, and its usefulness as an anti-Lp(a)-serum

The immune sera were tested by Ouchterlony's technique against the panel sera All immune sera precipitated all the human panel sera After 1–2 days, a spur could usually be seen between the precipitates against Lp(a+) and Lp(a—) human sera, indicating that the rabbit sera contained antibodies against an antigenic component present in the former, but not in the latter sera (see *Berg* 1964 b, Fig 3)

With several antisera an additional precipitation line near the antigen well developed against Lp(a+) human sera, instead of the described spur These findings indicated that the anti-Lp(a) activity was also present in unabsorbed immune sera

An experiment was performed to discover whether it was possible to perform tests for the Lp(a) factor by means of unabsorbed rabbit immune serum, taking advantage of the described spur or additional precipitate The immune serum was placed in the central well, one known Lp(a+) and one known Lp(a—) human serum distributed in two opposite peripheral wells, and the remaining four wells were filled with the human sera to be tested If a serum next to the known Lp(a+) serum was of type Lp(a—), or a serum next to the known Lp(a—) serum was of type Lp(a+), a spur would develop, whereas no spur formed if the neighbouring sera were of the same type In this way, 100 human sera of known Lp types were tested blindly for the Lp(a) factor The results were the same as those previously obtained with the Lp typing reagent This proves that the anti-Lp(a) activity is present in unabsorbed immune serum

Demonstration of the Anti-Lp(a) Activity by Intrabasin and Intra Gel Absorption of the Immune Sera on Agar Slides

All immune sera were tested against the panel sera after an intrabasin absorption with Lp(a—) and Lp(a+) human sera respectively 15 μl of the human serum was introduced into the central well and allowed to diffuse into the surrounding agar gel After about 2 hours the well was empty, and 15 μl of the immune serum was now placed in the same well At the same time, panel sera were filled into the peripheral wells The absorption of the unspecific antibodies then took place in the gel surrounding the central well and a circular precipitate could be seen around this well When the immune sera contained anti-Lp(a) antibody, they only reacted with Lp(a+) and not with Lp(a—) human sera when they were absorbed with a Lp(a—) human serum, whereas they lost their precipitating ability against all human sera when they were absorbed with a Lp(a+) human serum

The anti Lp(a) activity was not removed from an immune serum even if the well was filled several times with Lp(a—) human serum prior to the introduction of immune serum.

100 human sera of known Lp types were tested blindly against an immune serum absorbed by the intrabasin technique with equal parts of Lp(a—) human serum. No discrepancy as to the Lp types was observed. The intrabasin absorption technique can therefore be used for the demonstration of the Lp(a) factor.

A few immune sera were found which could not be rendered specific by absorption in tubes although the presence of anti Lp(a) antibodies could be demonstrated by the intrabasin absorption technique. These antisera are believed to be too weak to react after the dilution resulting from the absorption in tubes, whereas the intrabasin absorption takes place without dilution of the antiserum.

An experiment was also performed in which the human serum for absorption was mixed with the agar. Equal parts of molten 2 per cent agar in distilled water and Lp(a—) human serum heated on a water bath to 50° C were mixed and 2.5 ml of this mixture was poured on each of the 5 × 5 cm glass slides. When unabsorbed immune serum in the central well was tested on these slides against panel sera in the peripheral wells, a precipitate developed only against the Lp(a+) human sera. Experiments were also performed in which Lp(a+) human serum was mixed with the agar. No precipitates developed in these control tests. A circular precipitate was seen around the well containing the immune serum in these experiments.

From the experiments described in this section it was evident that the anti Lp(a) activity could be demonstrated without submitting the immune sera to the absorption procedure in glass tubes.

Absorption of Immune Sera in Tubes

In the absorption procedure originally used (*Berg* 1963), the mixtures of immune serum and Lp(a—) human serum were incubated for one hour at 37° C, the tubes tilted 10–15 times during this period and kept overnight at 4° C before centrifuging and testing of the supernatant.

To test the effect of absorption temperature and reaction time samples of one antiserum were absorbed with Lp(a—) human serum under the following conditions:

(1) Incubation for 1 hour at 37° C.
(2) „ 24 hours at 37° C.
(3) „ 1 hour at 4° C.
(4) „ 24 hours at 4° C.
(5) „ 1 hour at 37° C followed by 20 hours at 4° C.

The mixtures were centrifuged for 10 minutes at 1600 G immediately after the incubation period and the supernatants tested against Lp(a+) and Lp(a—) human sera.

No difference was found with regard to the results of the absorption in these experiments

It was thus obvious that other absorption procedures than the original one could also be used There is, however, no reason to change the absorption procedure, and the original one was therefore used as a standard in all later experiments

All immune sera were absorbed in glass tubes with different proportions between immune serum and Lp(a—) human serum, and thereafter tested against normal human sera For control, similar absorption tests were performed with Lp(a+) human serum It was found that the addition of a comparatively small amount of Lp(a+) human serum removed all precipitating ability from the immune sera, whereas the ability to react with Lp(a+) human sera usually remained over a wide range of absorption ratios with Lp(a—) human serum (see *Berg* 1963, Table 1)

Several rabbits immunized with whole human serum produced anti Lp(a) antibodies, as well as antibodies against various other serum proteins When antisera from such rabbits were absorbed with increasing amounts of Lp(a—) human serum, the last unspecific anti bodies to remain were anti-β-lipoprotein, sometimes together with anti α_2-macroglobulin, as shown by immunoelectrophoresis

Standard Lp typing reagents are prepared by absorption of rabbit immune sera in tubes Absorption ratios where equal parts of un diluted, absorbed immune serum and Lp(a+) human serum gave optimal reactions, were selected (see *Berg* 1964 c)

Before a new batch of absorbed immune serum was used as Lp typing reagent, the following control experiments were performed

5 μl of a Lp(a+) and the same amount of a Lp(a—) human serum were submitted to electrophoresis on the same slide, and allowed to diffuse against 100 μl of the absorbed immune serum A precipitate developed at the site of the β lipoprotein of the Lp(a+) human serum No precipitate could be observed between the antiserum and Lp(a—) human serum

Absorbed immune serum was tested in double diffusion experiments simultaneously against Lp(a+) whole serum and a solution of purified Lp(a+) β-lipoprotein A precipitate was formed against both these antigen solutions, and a reaction of identity was observed between the precipitates No precipitate developed when the antisera were tested against similar antigen solutions from Lp(a—) individuals

Lp(a+) human serum was tested simultaneously on Ouchterlony slides against verified anti Lp(a)-serum and apparently specific, absorbed immune serum A reaction of identity was always found between the precipitates arising from the two antisera, indicating that their specificities were identical

The precipitates in immunoelectrophoretic or double diffusion tests, between absorbed immune serum and solutions containing the Lp(a)

antigen were stained, and the presence of protein, lipid and esterase activity was demonstrated

To ascertain that the specificity of a new, absorbed immune serum was identical to anti-Lp(a), it was tested against several (at least 100) human sera which were already tested against an earlier produced anti Lp(a)-serum

Experiments Showing that the Loss of Precipitating Ability of an Anti Lp(a) Serum upon Addition of Excess Amounts of Lp(a—) Human Serum, Is only Due to Dilution

An anti Lp(a)-serum was diluted in different proportions in glass tubes with Lp(a—) human serum, and 0 85 per cent saline solution respectively The mixtures were treated in the same manner as in the standard absorption procedure No precipitation could be observed in the tubes Afterwards 15 µl of the mixtures was tested against equal amounts of Lp(a+) and Lp(a—) human sera No reactions against Lp(a—) sera were observed When 1 part or more of the anti-Lp(a)-serum was mixed with 1 part of either Lp(a—) human serum or 0 85 per cent saline solution, the immune serum still reacted in agar gel with Lp(a+) human sera No precipitate was formed when 1 part of the antiserum was mixed with 2 parts or more of either Lp(a—) serum or saline solution Thus the anti-Lp(a)-serum lost its precipitating ability at the same degree of dilution, whether Lp(a—) human serum or saline solution was used for the dilution

The result of this experiment indicates that the loss of precipitating ability, observed when unabsorbed immune serum is mixed with large amounts of Lp(a—) human serum in the standard absorption procedure, is an effect of dilution only

Attempts to Find other Specificities of the Immune Sera

It has been claimed (*Blumberg & Riddell* 1963) that several antigenic specificities exist within human β lipoprotein It is therefore possible that the rabbits might produce antibodies against other β lipoprotein factors than Lp(a), and that they could be immunized with whole human serum against other serum protein factors as well Several immune sera were absorbed with several different Lp(a—) or Lp(a+) human sera Absorption with Lp(a—) serum always resulted in an anti Lp(a)-serum if the immune serum contained anti-Lp(a) antibody, and absorption with Lp(a+) serum in complete loss of precipitating ability

Human serum from blood drawn at different times from the same individual, always had the same effect as absorption material

No difference was found with regard to the results of the absorption in these experiments.

It was thus obvious that other absorption procedures than the original one could also be used. There is, however, no reason to change the absorption procedure, and the original one was therefore used as a standard in all later experiments.

All immune sera were absorbed in glass tubes with different proportions between immune serum and Lp(a—) human serum, and thereafter tested against normal human sera. For control, similar absorption tests were performed with Lp(a+) human serum. It was found that the addition of a comparatively small amount of Lp(a+) human serum removed all precipitating ability from the immune sera, whereas the ability to react with Lp(a+) human sera usually remained over a wide range of absorption ratios with Lp(a—) human serum (see *Berg* 1963, Table 1).

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5 μl of a Lp(a+) and the same amount of a Lp(a—) human serum were submitted to electrophoresis on the same slide, and allowed to diffuse against 100 μl of the absorbed immune serum. A precipitate developed at the site of the β-lipoprotein of the Lp(a+) human serum. No precipitate could be observed between the antiserum and Lp(a—) human serum.

Absorbed immune serum was tested in double diffusion experiments simultaneously against Lp(a+) whole serum and a solution of purified Lp(a+) β-lipoprotein. A precipitate was formed against both these antigen solutions, and a reaction of identity was observed between the precipitates. No precipitate developed when the antisera were tested against similar antigen solutions from Lp(a—) individuals.

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antigen were stained and the presence of protein lipid and esterase activity was demonstrated

To ascertain that the specificity of a new absorbed immune serum was identical to anti Lp(a) it was tested against several (at least 100) human sera which were already tested against an earlier produced anti Lp(a) serum

Experiments Showing that the Loss of Precipitating Ability of an Anti Lp(a) Serum upon Addition of Excess Amounts of Lp(a—) Human Serum Is only Due to Dilution

An anti Lp(a) serum was diluted in different proportions in glass tubes with Lp(a—) human serum, and 0 85 per cent saline solution respectively The mixtures were treated in the same manner as in the standard absorption procedure No precipitation could be observed in the tubes Afterwards 15 μl of the mixtures was tested against equal amounts of Lp(a+) and Lp(a—) human sera No reactions against Lp(a—) sera were observed When 1 part or more of the anti Lp(a) serum was mixed with 1 part of either Lp(a—) human serum or 0 85 per cent saline solution the immune serum still reacted in agar gel with Lp(a+) human sera No precipitate was formed when 1 part of the antiserum was mixed with 2 parts or more of either Lp(a—) serum or saline solution Thus the anti Lp(a) serum lost its precipitating ability at the same degree of dilution whether Lp(a—) human serum or saline solution was used for the dilution

The result of this experiment indicates that the loss of precipitating ability observed when unabsorbed immune serum is mixed with large amounts of Lp(a—) human serum in the standard absorption procedure is an effect of dilution only

Attempts to Find other Specificities of the Immune Sera

It has been claimed (*Blumberg & Riddell* 1963) that several antigenic specificities exist within human β lipoprotein It is therefore possible that the rabbits might produce antibodies against other β lipoprotein factors than Lp(a) and that they could be immunized with whole human serum against other serum protein factors as well Several immune sera were absorbed with several different Lp(a—) or Lp(a+) human sera Absorption with Lp(a—) serum always resulted in an anti Lp(a) serum if the immune serum contained anti Lp(a) antibody and absorption with Lp(a+) serum in complete loss of precipitating ability

Human serum from blood drawn at different times from the same individual always had the same effect as absorption material

DISCUSSION

The experiments described demonstrate that the anti-Lp(a) activity of rabbit immune serum is caused by a specific antibody During the absorption with Lp(a—) human serum, antibodies against other serum proteins and against the β-lipoprotein antigens other than the Lp(a) factor are removed from the rabbit serum No unspecific absorption of anti-Lp(a) antibody takes place when large amounts of Lp(a—) human serum are added The eventual loss of precipitating ability is due solely to the effect of dilution of the antiserum

The absorption of the antisera in glass tubes is preferable to intra basin absorption, as the latter technique is more laborious and gives rise to circular precipitates around the antibody wells In addition larger amounts of standard typing reagent for the Lp tests can be prepared by the absorption in glass tubes

Whole human serum of type Lp(a—) is preferable as absorption material to a solution of isolated Lp(a—) β-lipoprotein, because many of the rabbit immune sera also contain antibodies against other serum proteins Immune sera from rabbits immunized with purified β-lipo protein instead of whole human serum can usually be absorbed with a β lipoprotein solution (see *Berg* 1963, Fig 4) It is, however, logical to use whole human serum for all absorptions, as serum is much more easily available than solutions of isolated β-lipoprotein

To obtain specific anti-Lp(a)-serum by absorption in glass tubes, it is essential that the immune serum contains anti-Lp(a) antibody in sufficient amounts to form a precipitate in agar gel with the Lp(a) anti gen, even after the dilution of the immune serum caused by this procedure Absorption by the intrabasin technique takes place without any dilution of the immune serum Therefore, some of the immune sera which could not be rendered specific by absorption in glass tubes could be used for Lp typing, when the intrabasin absorption technique was applied It is possible that immune sera of this kind could also be used for Lp tests after absorption in tubes, if the antibody wells were refilled so that considerably larger amounts of antiserum were used As it is fairly easy to produce antisera which are specific for the Lp(a) factor over a wide range of absorption ratios by the standard procedure in glass tubes, these weak immune sera were not used for Lp testing

Despite the fact that the anti-Lp(a) antibody lost its precipitating ability during the treatment with sodium sulphate, it was demonstrated, by the inhibition experiment, that the antibody belongs to the 7S γ-globulins of the rabbit Loss of flocculating ability with retained neutralizing ability after precipitation with sodium sulphate, has been reported for diphtheria antitoxin, and the possibility that this is due to dena turation of the antibody has been mentioned (*Boyd* 1956, p 327) It is possible that the loss of precipitating ability described for the anti-Lp(a) antibody is a parallel to this phenomenon

No systematic investigation of the stability of the anti-Lp(a) antibody has been made. Antisera, both absorbed and unabsorbed, have been kept at −25° C for 2 years, apparently without any deterioration of the anti-Lp(a) antibody.

So far, no antibodies against other individual specific antigenic factors than the Lp(a) have been detected in the immune sera. A systematic search for such antibodies was, however, not the purpose of the present work, and has not been done. If antibodies against other individual antigenic factors were present in the sera, they would probably have been revealed by the tests already performed. The rabbits apparently form precipitating antibodies against the Lp(a) factor more readily than against any other individual specific antigenic factor of human serum.

SUMMARY AND CONCLUSIONS

1. The anti-Lp(a) activity is caused by a specific antibody present in the rabbit immune sera.
2. The anti-Lp(a) antibody belongs to the 7S γ-globulins of rabbit immune serum.
3. The immune sera are rendered specific for the Lp(a) factor by absorption with Lp(a—) human sera.
4. A standard absorption procedure in glass tubes is performed to prepare anti-Lp(a)-sera for testing.

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Department of Biophysics Statens Seruminstitut Copenhagen Denmark

HYPO-7S-γ-GLOBULINAEMIA IN MATURE CATTLE

By

B Mansa

Received 16 vii 64

Immunoelectrophoretic analysis of human sera (5) and different animal sera has shown three main components of immune globulins, i e γ_{1A}, γ_{1M} and 7S γ globulin (9, 2, 4, 6 8) The relative intensities of the γ_{1A} and 7S γ globulin lines vary from species to species (12) Thus in contrast to human serum, where the 7S-γ-globulin is the main constituent γ_{1A} globulin dominates in serum from cattle The present study reports on hypo 7S-γ globulinaemia in mature cattle as revealed by immunoelectrophoretic investigations

MATERIAL AND METHODS

Serum samples from 780 head of cattle at least one year old were investigated The animals belonged to 45 herds on the islands of Lolland and Falster and with the exception of 20 animals were of the Red Danish Milk Breed

Immunoelectrophoresis was carried out according to the method described earlier (11) with minor modifications (10) A pool of rabbit antiserum against normal bovine serum was used as well as a series of individual rabbit antisera of the same specificity The pool was absorbed for antibodies against γ_{1A} globulin as described below

EXPERIMENTS AND RESULTS

Undiluted sera from the 780 head of cattle[1] were investigated by immunoelectrophoretic analysis with the use of unabsorbed pooled antiserum The slowest migrating immune globulin (7S-γ globulin) could not be demonstrated in 22 of the sera while a faint 7S γ-globulin precipitation line, developed by excess of antibody indicated subnormal amounts of this component in 107 sera The remaining 651 sera showed a strong 7S γ globulin line developed by excess of antigen Examples of the different groups of sera are shown in Fig 1 The γ_{1A} globulin precipitation lines indicated only slight variations in γ_{1A} globulin concentration in the total number of sera Consequently, with respect to this constituent they were all regarded as normal

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[1] The author wishes to express his gratitude to Dr *K Fennestad* The Leptospira Department Statens Seruminstitut who kindly placed the sera at my disposal

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Fig 1
A Immunoelectrophoretic analysis of normal cattle serum
B D Examples of sera which show subnormal amounts of 7S γ globulin
(the slowest migrating component)
Antiserum Pooled rabbit antiserum against normal bovine serum

A series of absorption experiments was performed on the antiserum pool using increasing concentration of one of the first mentioned 22 sera for removal of antibodies When 20 μl of serum per ml of antiserum was used, all antibodies against γ_{1A}-globulin were removed, while some of the antibodies against 7S γ-globulin still remained The concentration of the 7S-γ globulin antibodies had distinctly decreased after the absorption due to some common antigenic sites of these two types of immune globulins Simultaneously, the concentration of antibodies against other components had considerably decreased Concerning the electrophoretically slow moving serum components, the absorbed antiserum could be regarded as specific for the 7S-γ-globulin By means of this absorbed antiserum, analysis of 54 normal sera taken at random all showed a broad 7S-γ globulin line typical of excess of antigen

The selected 129 sera were subjected to a second immunoelectrophoretic examination with the aid of this absorbed antiserum Even then 7S-γ-globulin could not be demonstrated in 8 sera belonging to the previously mentioned group of 22 which was found to be devoid of 7S-γ-globulin However, the remaining 121 sera all showed a 7S-γ globulin precipitation line The intensity of these lines covered the range from a barely visible short line developed by antibody excess, to a normally extended line in the zone of antigen antibody equivalence



Fig 2

Immunoelectrophoretic analysis of the same serum samples as in Fig 1 presented in the same sequence The antiserum was absorbed prior to the application for removal of antibodies against γ_{1A} globulin as described in text The remaining anti 7S γ globulin antibodies demonstrated the presence of small amounts of 7S γ globulin in the sera shown in C and D while 7S-γ globulin could not be demonstrated in the serum shown in B Note the 7S γ globulin precipitation line developed by antigen excess in normal serum (A)

(Fig 2) Thus the 7S γ-globulin lines were clearly distinguishable from those of normal sera

Absorption experiments were repeated by the use of other sera belonging to the group of 8 In addition a series of individual antisera were absorbed in order to produce specificity for the 7S γ-globulin Qualitatively reproducible results were obtained from all of these experiments

Serial two fold dilutions of ten normal sera were examined by immunoelectrophoresis against an absorbed antiserum The relative 7S-γ-globulin concentrations in the selected 121 sera were roughly estimated from the appearance of this arc, in comparison to those developed by the diluted sera A concentration up to ten times lower than that of normal sera was found in 72 sera, and ten to fifty times lower in 49 sera Furthermore the 8 sera which did not show any 7S γ-line, could contain at a maximum one hundredth of the amount present in normal sera An example of a normal serum diluted one in ten and one in twenty is shown in Fig 3

Paper electrophoretic analysis (1) on 54 of the selected sera showed γ_{1} + γ-globulin concentrations varying from 14 to 30 per cent of the total proteins The sera are thus undistinguishable from normal sera by

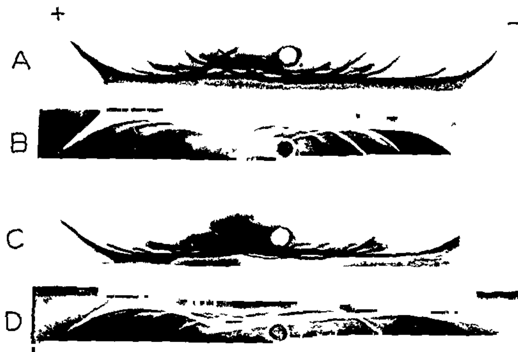


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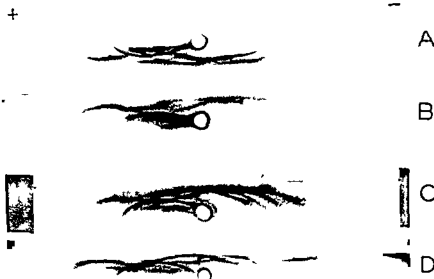


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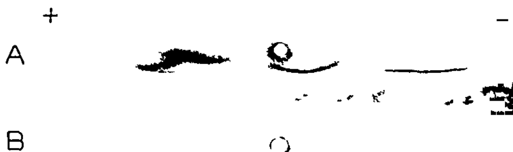


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Immunoelectrophoretic analysis of normal bovine serum diluted one in ten (A) and one in twenty (B) The same antiserum as in Fig 1 was used

this method (15, 16) Similar results were obtained for the concentration of the 7S-globulin component determined by analytical ultracentrifugation (11 to 33 per cent)

DISCUSSION

When immunoelectrophoresis on sera from mature cattle was carried out by the aid of pooled antiserum against normal bovine serum, deviating patterns indicating subnormal concentrations of the 7S γ globulin were found in 15 per cent of the animals This differentiation was possible provided the antiserum used contained sufficient amounts of antibodies specific for 7S-γ-globulin, in addition to those specific for γ_{1A}-globulin With such an antiserum the slowest migrating part of the two globulins are precipitated separately This condition was generally fulfilled by the antisera used in this investigation, but a few antisera presenting a common precipitation line have been encountered

As the γ_{1A} and the 7S γ-globulin lines fused within their intermediate mobility ranges, a specific anti 7S-γ-globulin antiserum was needed to avoid the masking effect of γ_{1A}-globulin This was obtained by absorption of antiserum with one of the hypo-7S γ globulinaemic sera Antibodies against faster migrating components which partly remained after the absorption apparently did not interfere

The 8 sera, in which it was impossible to demonstrate 7S-γ-globulin, are essentially hypo-7S-γ-globulinaemic, as they could contain at a maximum one hundredth of the amount estimated as normal A still more sensitive method would probably reveal small traces of 7S-γ globulin also in these sera Despite the possibility of differentiating between normal sera and the remaining 121 sera of the selected group, a clearcut lower limit of the normal 7S-γ-globulin concentration cannot be given The subnormal concentrations are distributed within a rather wide range, the upper part of which shows rather close approximation with the lower part of the normal range The influence of differences in titres against γ_{1A}- and 7S γ-globulin of the antisera for the first

selection contributes also to the difficulties in obtaining a lower limit for the normal 7S-γ globulin concentration

Originally the sera were collected for an investigation of Leptospira antibodies Agglutinins against one or more of ten Leptospira serotypes known to occur in Denmark were evenly distributed among the normal and the hypo γ globulinaemic sera Thus it was impossible to demonstrate any correlation between the presence of Leptospira agglutinins and the type of hypoglobulinaemia studied

γ_{1A} globulin is considered the main immune globulin in cattle sera (12) The electrophoretic mobility of this component (14) as well as the value of its sedimentation coefficient are found to be very similar to those of the 7S γ globulin (7 13) The results of paper electrophoresis and analytical ultracentrifugation experiments presented in this report corroborate these previous findings

The hypo 7S-γ globulinaemia is presumably a result of subnormal production of the 7S γ component Any relation to the hypo-γ globulinaemia seen in 10 0 per cent of suckling calves and recently reported (3) is unlikely after taking into consideration the different origin of the immune globulins in newborn calves and in mature cattle

SUMMARY

Sera from 780 mature Danish head of cattle were investigated by immunoelectrophoresis With a rabbit antiserum against normal bovine serum it was impossible to demonstrate the presence of the slowest migrating immune globulin (7S γ globulin) in 22 of the sera A faint 7S γ globulin precipitation line indicated subnormal amounts of 7S γ globulin in 107 sera

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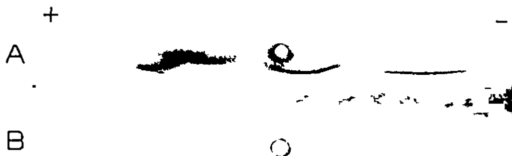


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BRIEF REPORT

EFFECT OF IMMUNIZATION WITH HOMOLOGOUS RETROBULBAR TISSUE EXTRACTS ON THE DEVELOPMENT OF EXPERIMENTAL ENDOCRINE EXOPHTHALMOS

By C Friman E J Jokinen R Stenstrom and O Wegelius

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The pathogenesis of endocrine exophthalmos is not yet fully understood It may be regarded as establised however that a hypophyseal factor EPS (exophthalmos producing substance) and possibly also a substance of hypothalamic origin LATS (long acting thyroid stimulator) are of fundamental importance for the development of the condition

Tissue infiltration of lymphocytes is a characteristic feature of the morphological changes observable in endocrine exophthalmos (*Smelser* 1937, *Wegelius et al* 1957)

autoimmunization process In this connexion it is noteworthy that the concurrence

in estigate I According to our knowledge no experimental investigations regarding the role of immunological factors in the development of endocrine exophthalmos have been performed Hence a pilot study in this field was considered of some interest

Material and Methods

Seventy five male guinea pigs weighing 250–300 g were divided into 6 groups and treated as listed below

Group	No of animals	Procedure
I	10	TSH
II	15	TSH after thyroidectomy
III	10	Homogenate + Freund s adjuvant
IV	10	TSH + homogenate
V	15	TSH + homogenate after thyroidectomy
VI	15	0 9 per cent NaCl

TSH Organ n) was ... mg for 12 days ... homogenizing ... in 0 9 per cent ... eraperitoneally every second ... interval of 2 weeks Homo

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STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

5 *The Induction of Tolerance to the Virus*

By

MOGENS VOLKERT and JØRGEN HANNOVER LARSEN

Received 15 ix 64

The induction of immunological tolerance to non viral antigens has been shown repeatedly to depend on the maturity of the animals employed and on the antigenic dose As regards the mouse the first few days after birth are crucial (1) Within this period injections of small antigenic doses are sufficient to induce a state of tolerance wheras much higher doses must be employed in older animals Furthermore it has been demonstrated in transplantation immunity studies that the genetic (antigenetic?) relationship between host and donor plays an important role for the induction and duration of tolerance to cellular antigens As regards the induction of a state of tolerance to the LCM virus *Hotchin & Cinits* (6) were the first to show that tolerance could be induced in mice if the animals were inoculated with the virus shortly after birth However it seemed to be difficult to induce tolerance to the LCM virus in older animals The results of *Hotchin & Cinits* have been confirmed by others (16 18) but a more thorough investigation of the matter has not been carried out Moreover tolerance to the LCM virus has not been observed in other animal species than mice but no attempts to elucidate this problem have been reported

As the question of tolerance to a virus might have important implications (18 19) it seemed appropriate to investigate the mechanism of the induction of this immunological phenomenon It is the purpose of this paper to present the experimental results of attempts to induce immunological tolerance to the LCM virus in mice and some other animals

MATERIAL AND METHODS

The virus and the *serological procedure* were as described in previous reports (18 19 20 21)

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genate in combination with Freund's adjuvant (Difco) were mixed in the proportion 1 2 Thyroidectomy was performed by injecting 468 μc of I^{131} intraperitoneally 2 weeks before immunization was commenced Exophthalmos was measured radiologically according to *Tengroth* (1961) with minor modifications The occurrence of serum antibodies was investigated by 3 methods 1 Passive hemagglutination (*Boyden* 1951) using phosphate buffer extracts (PH 7 2) of normal eye muscle lacrymal gland and Harder's gland as antigens 2 Complement fixation using borate buffer extracts (PH 7 0) of the same tissues as antigens 3 Complement fixation (1 5 volume Kolmer technique) using ethanol extracts of the above tissue as antigens Retrobulbar tissue specimens were histologically studied after formalin or lead acetate fixation and staining with haematoxylin eosin or toluidine blue

Results

As was to be expected significant exophthalmos resulted in group II in all animals but one In groups IV and V interpretation of the radiological results was hampered by high mortality towards the end of the experiment but the values obtained speak against an enhancement of exophthalmos resulting from immunization

No circulating antibodies were demonstrable in the immunized animals by any method

Histologically a varying degree of cellular infiltration was found in the TSH treated groups No definite effect on the retrobulbar morphology was attributable to immunization

Under the present experimental conditions immunization with retrobulbar tissue extracts did not enhance experimental exophthalmos or related morphological changes in guinea pigs The results confirm earlier studies on humans in which no antibodies against retrobulbar tissues were demonstrable in patients with endocrine exophthalmos

In human malignant exophthalmos the possibility remains however that a forbidden clone (*Burnet* 1962) of immunologically competent cells is of pathogenetic significance Such a genetically or mutationally conditioned immunological disturbance is difficult to produce experimentally though If future immunological studies of endocrine exophthalmos do not disclose evidence contrary to that obtained so far the interesting possibility remains that the morphological changes discernible in this condition are solely due to an endocrine effect

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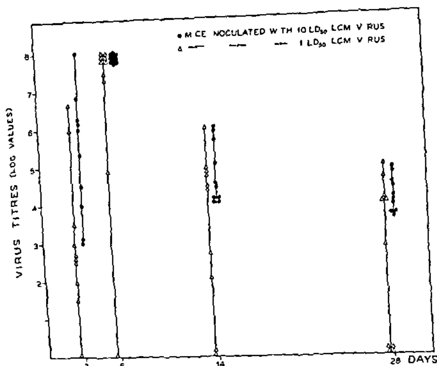


Fig 1

Blood virus titres of AkA mice following infection initiated < 18 hours after birth with either 10 or 1 LD50

100 newborn mice were employed Most of them were killed in groups at different intervals after the infection Thirty were kept alive and bled at one or two week intervals from the age of two weeks As expected it was found that the percentage of mice in which virus could not be detected in the blood varied greatly from experiment to experiment and also from litter to litter Use of inbred mice of strain AKA and a very carefully titrated virus batch diminished the variations Under such experimental conditions it was observed that the number of virus free mice increased considerably with time and was several times higher at the age of 4 6 weeks than at the age of 3 6 days (8 out of 20 and 2 out of 20 respectively) Such data suggests that some mice in which the virus is found initially loose their virus in the course of some weeks More direct evidence of this assumption has been obtained in two mice only Both of these mice had virus in their blood two weeks after they as newborns were injected with 1 LD50 dose No trace of virus was found in their blood at the age of two months

The extended experiments confirmed that some and even most of the mice which were given 1 LD50 of virus had a slow increase in the virus titres of the blood (Fig 1) This was clearly demonstrated by the virus

The blood was taken from the heart or from the retro bulbar veneous plexus. Virus titrations (beginning with a dilution of 1/10) could be carried out on blood samples from individual live two week old mice. Enough blood for both a virus titration and a complement fixation test could usually be obtained when the mice reached the age of four weeks. (The virus titrations beginning with a blood dilution of 1/10 and the complement fixation test with a serum dilution of 1/4 in 0.1 ml amount).

Animals. The mice for the tolerance studies belonged either to inbred strains of AKA and C_3H mice (18–20) or to the institute's stock of ordinary white Swiss mice. Only the latter strain was used for virus titration purposes. The hamsters, guinea pigs, rabbits, rats, and chickens were not inbred but all originated from the stocks of these animals kept at this institute.

EXPERIMENTAL

Induction of Tolerance to the LCM Virus in Newborn Mice

Growth curve of the virus in neonatally infected mice: As the virus is a self-replicating agent, the amounts of antigen to which an animal is exposed at a given time must depend not only on the dose of virus injected but also on the rate of growth of the virus. For that reason and because of the fact that especially the newborn mice are of interest in tolerance induction experiments, the growth of the virus in such animals was investigated. Groups of newborn mice less than 18 hours old were inoculated intraperitoneally with 0.03 ml doses of LCM virus in varying dilutions. Three, six, nine, 14 and 28 days after the infection, some mice (usually 10) from each group were killed and the blood from the individual mice tested for virus. Moreover, in one experiment the mice were allowed to live but the blood was tested for virus at one to two-week-intervals from the age of 2 weeks. The results demonstrated clearly that all the mice which received 1000, 100 or 10 LD_{50} developed viraemia, and the virus titres of the mice in the different groups were within the same range at various times, irrespective of the infection dose. Fig. 1 records a representative experiment and shows the titres of a group of mice infected with 10 LD_{50}. Within three days the virus titres in the blood had reached a titre of at least 10^{3} and often higher, and on the sixth day all had blood virus titres $\geqq 10^{6}$. The titres remained at this high level for about a week and then declined rapidly. Already on the 14th day many of the mice had reached a blood titre of 10^{4}. At that level the virus remained constant not only until the 28th day but throughout an observation period of many months.

When newborn mice were infected with 1 LD_{50} of virus, many of these showed the same virus pattern in their blood as described above. However, some mice reacted differently. First of all it was found that a rather large percentage of the infected mice did not have a detectable amount of virus in their blood either shortly after the inoculation or later on. Furthermore, some of the mice in which the virus did appear in the blood seemed to have a slower virus growth rate than the mice infected with 10 or more LD_{50} of virus. In order to obtain detailed information about these observations, the investigation on the course of infection initiated by virus doses of 1 LD_{50} was extended. More than

those found in tolerant, serologically non reactive, virus carriers (18, 20)

Of five mice which showed viraemia after infection with 0 1 LD_{50} one developed complement fixing antibodies and these antibodies were also present together with a high virus titre Attempts have been made to follow the virus titres in serologically positive viraemic mice Up to now it has not been possible to demonstrate disappearance of virus in such mice

Conclusion All newborn mice infected with 10 or more LD_{50} of virus showed a rapid virus growth rate, developed persistent viraemia and did not develop antibodies *i e*, the mice were rapidly exposed to high virus doses and were made tolerant to the virus When infected with 1 LD_{50} a slow growth rate of the virus occurred in certain mice and some developed antibodies, *i e*, some mice were–at least in the beginning–exposed to small antigenic doses and some became actively immune (Table 1 and 2)

TABLE 2

Importance of the Infection Virus Dose and the Age of the Mice on Induction of Tolerance to the Virus

Virus dose	Age when infected	1 week old + Viraemia	4 weeks old + C F† + Viraemia		6 weeks old + C-F + Viraemia		12 weeks old + C-F + Viraemia	
0 1 LD_{50}	< 18 hours		0/22	5§/22	1/5	5/5	0/4	4/4
1 LD_{50}	< 18 hours	18/20	0/20	12 20	3/16	10/16	3/16	10/16
10 LD_{50}	< 18 hours	20/20	0/14	14/14	0/13	13/13	0/13	13/13
100 LD_{50}	< 18 hours	20 20	0/20	20/20	0/18	18/18	0/17	17/17
100 LD_{50}	4 days		0/22	22/22	6/20	22/22	5/13	11/13
100 LD_{50}	8 days		0/14	14/14	12/14	13/14	8/10	4 10

Virus titres > 2 5 [illegible]

[illegible] 6th and 12th week

Induction of Tolerance to the LCM Virus in Baby Mice and in Mature Mice

Traub (15) and *Hotchin & Weigand* (7) have shown that the pronounced resistance of newborn mice to intracerebral injections of the LCM virus is lost within a few days after birth However, in the present study baby mice at the age of four and even eight days showed quite good resistance to intraperitoneal injections with virus doses of 100 LD_{50} As reported above such a virus dose in the newborns is more than enough to secure the induction of tolerance in all cases Therefore, 100 LD_{50} of the LCM virus given intraperitoneally to baby mice at different ages should reveal the importance of the age factor in inducing tolerance to the virus The results of intraperitoneal injection into baby mice four

titres on the third day after infection. Already on the sixth day, however, the virus titres had reached values equal to those obtained when large infection doses were used. Later most of the mice also had titres equal to those in the other groups. In a few cases, however, the late blood titres were exceptionally low, so low that they might indicate that the virus in the blood of these mice was about to disappear.

Experiments using infection doses of less than 1 LD_{50} were attempted but very few mice became infected and blood virus curves could not be obtained.

TABLE 1

LCM Virus and Antibody Titres in Newborn Mice Infected with 1 LD_{50}

Mouse No	Age in weeks	CF titres	Virus titres (log values)
1 2 3	4	8	≥ 3.5
4	8	9	3.5
5	12	8	≧ 3.5
6	5	16	4.5
7	8	32	3.5
8	6	128	≧ 3.5
9	6	256	2.8
10 11	6	16	n tr
12	8	16	
13	4	32	
14	6	32	
15	12	32	
16	4	64	
17	6	64	
18	8	64	
19	9	128	
20	16	128	
21	6	256	
22	11	256	

n tr = no trace

Appearance of complement fixing antibodies. The blood from the mice in the above mentioned experiments was also tested for complement fixing antibodies. It was found that in none of the mice which received virus doses of 10 and more LD_{50} were antibodies detectable at any time. As regards the mice given 1 LD_{50}, no antibodies were demonstrated in the first two weeks. Later, however, from the fourth to the eighth week, roughly *20 per cent developed complement fixing antibodies* to the LCM virus. Table 1 records the maximum titres obtained in the 22 mice which reacted positively with the virus antigen. It is apparent that titres as high as 128-256 were not uncommon. In most cases where antibodies were found, no virus was present in the blood. However, it is very striking that in as many as one third of the serologically positive mice virus and antibodies were found together. Moreover, the virus titres in the serologically positive mice were as high as

blood However, from then onwards the titres declined Some individual variations were observed, but at the age of three months eight out of 11 hamsters had no detectable virus in their blood and the remaining three had only traces The blood of the hamsters was also tested for complement fixing antibodies During the first three weeks after the infection none of the animals developed antibodies, but from then onwards more and more were found to be serologically positive, and at the age of six weeks all had complement-fixing antibodies to the LCM virus The titres were usually 128–512 As in the case of newborn mice infected with 1 LD_{50} of virus, in the beginning complement-fixing antibodies and high blood titres ($\geqq 10^{3.0}$) were very often found together Later on only the antibodies persisted

Rabbits Newborn rabbits were found to be very resistant to the virus In no case was any animal killed by the virus, even when the highest infection doses were employed Of nine animals infected intraperitoneally with 10^{6} LD_{50} at birth and killed two months later, none had viraemia and only two had small amounts of virus in the spleen and kidney All animals developed complement-fixing antibodies but the titres were modest – usually 32

Guinea-pigs Newborns were very susceptible to the virus and in all experiments all the infected animals died within two weeks even when very small infection doses were employed

Rats Newborn rats and baby rats up to the age of 5 days were also highly susceptible to the LCM virus However, from the age of about a week the animals began to develop resistance and at the age of three weeks they survived any virus dose given intraperitoneally Sixteen rats surviving intraperitoneal infection with 10^{5} LD_{50} at the age of one week were observed for three months At the age of four weeks 12 of the animals had detectable virus in their blood The titres were not high, maximum $10^{2.0}$ At the age of three months no virus could be detected in the blood of any of the animals Complement-fixing antibodies were found as early as three weeks after the infection and were present in all at the age of three months Titres of 512 were very common Infection of rats older than two weeks has also been investigated All the animals tested so far had no virus in their blood three weeks after the infection and all had complement-fixing antibodies at that time

Chickens Chick embryos 7 and 11 days old were infected through the yolk sac with varying doses of virus All died, even when doses as small as 1 LD_{50} were employed However, at the age of 15 days the embryos had developed resistance to the virus All of 10 embryos of that age survived 10^{5} LD_{50} and hatched normally On hatching three were tested for virus Two of these had no detectable virus either in the blood or in the organs In one a trace of virus was detectable in the spleen and the kidneys At the age of two months no trace of virus was found in blood or organs of the five chickens which were alive and tested at that time

and eight days old with 100 LD_{50} of virus are recorded in Table 2 It is apparent that all the four days old mice developed viraemia (titres $\geqq 10^{3.5}$) However, from the age of about four weeks complement fixing antibodies could be found in about one-third of the mice and in some the virus in the blood disappeared As in the case of newborns injected with small virus doses, it was not uncommon that antibodies and virus in apparently undiminished titres were found together in the same blood samples When baby mice of the age of eight days were employed in the study, all the mice developed also viraemia with high virus titres, but in the course of 12 weeks about two thirds of these mice had complement-fixing antibodies and in about one-third virus was no longer detectable in the blood These results demonstrate that already at the age of four days, and later to an even greater extent, a virus dose of 100 LD_{50} was not sufficient to induce tolerance in *all* infected mice On the other hand, as many mice, even in the eight days age group, were found to be persistent virus carriers without an immunological response to the virus it is still possible to induce tolerance at that age As in most cases the appearance of antibodies was delayed, a temporary state of tolerance seems to be common in this type of experiment

Mature mice are usually also resistant to intraperitoneal injections of 100 LD_{50} of the strain of LCM virus employed in this laboratory For the purpose of producing immune donors for transplantation studies, many dozens of inbred mice of strain AKA and C_3H have been infected intraperitoneally with 100 LD_{50} of virus and in many cases the mice have been tested for virus and antibodies 3–4 weeks later In all cases antibodies were present, in most cases the blood was free of virus and in a few cases only traces of virus were detectable A state of tolerance was never induced

Attempts to Induce Tolerance to the LCM Virus in Animals other than Mice

The LCM virus is pathogenic for many animal species, including man (4, 9, 10, 13, 14) In the experiments in this study newborn hamsters, rabbits, guinea-pigs, rats, and chick embryos were infected in attempts to induce a state of tolerance to the virus

Hamsters Preliminary experiments revealed that in many cases newborn hamsters could survive intraperitoneal injections of up to 10^5 LD_{50} of virus Accordingly, 10^5 LD_{50} of virus were given intraperitoneally to newborn hamsters In all 33 animals were infected Within the first week, all the animals had high virus titres in their blood ($\geqq 10^{3.5}$) Within the following two weeks many of the animals became sick and 22 died The rest recovered and survived throughout an observation period of three months Of the survivors, nine were tested for virus at the age of four weeks and all of these still had very high titres in their

that a state of tolerance changes spontaneously to a state of immunity However, it is generally agreed that tolerance prevails as long as the inducing antigen persists in amounts above a certain critical level, and the spontaneous abolition of tolerance is a consequence of antigen reduction However, this does not always seem to be the case when the antigen is the LCM virus The observation that antibodies are present together with virus titres as high as those seen in mice remaining tolerant indicates that the abolition of tolerance can take place before a decline in antigen has occurred, or at least has reached below the level which in other mice is sufficient to maintain a tolerant state Why this happens is a mystery However, it might also be a question of age and antigenic dose Perhaps it is so that all immunologically competent cells can be made tolerant only if enough antigen is present at a very early state of life If this is not the case, or if the inducing antigen is given too late a few cells might escape being made tolerant It might be also assumed that these cells multiply and are sensitized by the virus antigen However, the immunity caused by them will not be observed until they have increased sufficiently in number so as to make their activity detectable Therefore, the temporary state of tolerance observed in the first few weeks may be only an apparent phenomenon It is difficult to understand why the cells which fail to become tolerant in the first few days after birth are not paralyzed later or made tolerant by the huge amount of virus present Probably these cells are different Perhaps it is a question of maturation, and perhaps this maturation does not occur until some days after birth or it may never occur in the presence of excess of antigen We do not know It might be so and th[illegible]

[illegible] tolerant (a) On the other hand, it seems to contradict the assumption that the induction of tolerance is just a matter of the quantity of antigen and the number of cells exposed (8, 11)

The attempts to induce tolerance in animals other than mice are too few to exclude the possibility that it is practicable However, they demonstrate clearly that it is very difficult and that the state of tolerance to the LCM in other animals if existing, must be very rare The reason for the negative results obtained when tolerance is induced in newborn rats and guinea pigs and in chick embryos might be that infection with the virus is lethal in these animals However, this is not so as far as hamsters are concerned In spite of the fact that the virus grows in high titres in the newborns of these animals, a persistent state of tolerance was not induced This might be due to a slow growth rate of the virus in the newborn hamsters It might perhaps also be due to lack of antigenic compatibility of the virus and its host We are most inclined to accept the last possibility Judging from the results obtained in transplantation immunity studies the antigenic factor of both the

Conclusion: A state of tolerance to the LCM virus was not induced in either rabbits, guinea-pigs, rats, or chickens In hamsters a temporary state of tolerance seemed to be present at first but did not last long and in all cases was followed by the development of active immunity

DISCUSSION

As expected, the experiments have demonstrated that the age of the mice is of great importance for the induction of tolerance to the LCM virus Even as early as four days after birth many mice will not be made tolerant by injections of virus doses which would secure tolerance in newborns To induce tolerance in older baby mice is more difficult and has not been achieved in mature mice Instead of inducing tolerance in mice at the age of four days or older, injections of virus may cause active immunity However, active immunity can also be induced in newborn mice if sufficiently small doses of virus are injected Study of the growth of the virus in newborn mice revealed that in certain mice virus doses of about 1 LD_{50} did not cause viraemia thus indicating that only very modest virus multiplication had taken place That such mice are made immune instead of tolerant is very understandable Probably the antigenic dose does not reach above the level critical for the induction of tolerance In other newborn mice infected with 1 LD_{50} of virus, viraemia did occur and maximum blood virus titres were obtained Nevertheless, some of these mice also developed antibodies The fact that the growth rate of the virus in newborn mice infected with 1 LD_{50} showed great variations and in certain animals was rather slow gives an explanation of these results Most probably the viraemic mice which became actively immune are those in which the virus multiplied slowly In these latter, the virus does not reach a high titre until about a week after the infection As the age factor is important, this might mean that within the first critical period after birth the antigenic mass of the virus does not reach the level necessary for an induction of persistent tolerance When the virus finally reaches a high titre it is too late, and the mouse is too old to be made persistently tolerant by that amount of virus

All of the mice, infected neonatally with small virus doses and in which virus was found in the blood, developed their viraemia within the first two weeks This observation, together with the fact that antibodies to the virus were not detected in any case until the mice had reached the age of 4–6 weeks, at which stage they in many cases were found together with the virus indicates, that in some mice a temporary state of tolerance was present initially, later to change spontaneously into a state of active immunity The same seems to occur when four and eight days old mice are infected with large doses of virus

In studies of tolerance to other antigens it is a common observation

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donor and the host is of the greatest importance for the induction of tolerance to tissue grafts

The resistance of the immunologically non-responsive newborn mice to the LCM virus, together with other striking observations made *Rowe* (12) and *Hotchin* (5) suggest the hypothesis of the virus itself being harmless and that it was rather the immune response which caused disease However, the experiments demonstrating the lethal effect of the virus in newborn rats, and in young chick embryos (animals which must be considered even more immature and less immunologically responsive than newborn mice) contradict this assumption, at least when animals other than mice are concerned

SUMMARY

A state of persistent immunological tolerance to the LCM virus is induced in all newborn mice by infection of these with 10 or more LD_{50} of virus Small doses of virus can also induce tolerance in newborn mice However, in certain of these cases the multiplication of the virus is too low or the virus growth rate too slow to establish a state of persistent tolerance Under such conditions an active immunity is to develop later in life

Four and eight days old mice infected with 100 LD_{50} are either made tolerant or actively immune A temporary tolerance may also occur Mature mice have not been made persistently tolerant to the LCM virus

In hamsters a temporary state of tolerance can be induced by infection of the newborns

It has not been possible to induce tolerance to the LCM virus in rats, guinea-pigs, rabbits, or chickens

The change from tolerance to immunity is discussed

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forces Since such an achievement would be highly desirable, the effect of immune serum and immune homologous lymphoid cells on tolerance to the LCM virus was reinvestigated It is the purpose of this paper to present the results of these experiments

MATERIAL AND METHODS

Th...

after transplantation of 100 × 10^6 immune isologous lymphoid cells Serum with lower titres was obtained from the same kind of mice but at a later time after transplantation

Mice tolerant to the LCM virus were all kindly inbred and belonged either to strain AKA or C_3H Tolerance to the virus was brought about by infecting the newborns with the ...

... from the other mice the cells were harvested two or three weeks after the last virus injections Both types of donors had previously been found to be highly immune (5)

Preparation of cell suspensions transplantations serological and other methods have been described in previous publications in this series (4 5 6 7,8)

EXPERIMENTAL

Effect of Immune Serum

Antibodies conferred to normal mice Experiments were first carried out to determine the antibody titres and the persistence of antibodies in the blood of normal mice after injection of immune serum Five AKA mice weighing 12–15 g were employed The serum batch used had a complement fixing antibody titre of 1024 and was given intravenously in amounts of 0 5 ml Under these experimental conditions it was found that 24 hours after the serum treatment the antibody titres in the blood of the recipients varied from ½ to ¼ of that in the injected serum The mean titre was 512 The half life of the complement fixing antibodies was about 4 days, *i e*, almost all the antibodies had disappeared within a month (Fig 1)

Antibodies conferred to tolerant virus carrier mice From the experiments just described it seemed possible to obtain and maintain high antibody titres in normal mice by injection of immune serum However, this would not necessarily be so in virus carriers In these an ... large ... haps ... body ... this problem the experiments with immune serum were repeated in six AKA virus carriers On the whole, the results were identical with those described above As will be seen in Fig 1, the antibody titre curves obtained after injection of serum were completely normal On the other hand, the initial titres obtained 24 hours

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STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

6 *Immunity Conferred on Tolerant Mice by Immune Serum and by Grafts of Homologous Lymphoid Cells*

By

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Received 21 ix 64

The workers in the field of tissue transplantation immunity agree generally that the abolition of a state of tolerance brought about by transplantation of lymphoid cells is caused by repopulation of the new host with the transplanted cells and by the persistent active immunity conferred by these cells (1, 3) A conferred immunity which does not last should not terminate a state of tolerance Accordingly, injections of immune serum and transplantations of non-compatible cells have been demonstrated repeatedly to be useless procedures in tolerance abolition experiments (2) It seems logical to assume that the same should be the case where tolerance to a virus is concerned Preliminary investigations with tolerant LCM virus carrier mice (4, 5, 7, 8) have already given results which strongly support that assumption However, the dose and potency of immune serum used in the virus experiments were only modest, and from the experiments where the homologous cells were employed, only the negative effect of one, rather small dose is known Theoretically at least, the possibility still existed that higher doses of more potent sera or large grafts of homologous lymphoid cells might alter the results It is obvious that the immunity conferred by immune serum or by homologous lymphoid cells would be temporary only in all cases However, it could not be excluded beforehand that if this immunity was strong enough, the virus titres in blood and organs might be depressed Perhaps the virus antigenic mass could be reduced so much that it was brought below the level necessary for maintenance of tolerance If this could be achieved and the virus kept down for a time sufficient to give the lymphoid cells of the tolerant animals a chance to maturate without being destroyed or made tolerant, then restoration of the immune mechanism might perhaps be the result The new immunologically competent cells could be expected to be sensitized by the antigen still present, an active immunity might develop, and the tolerance be brought to an end by the animals' own

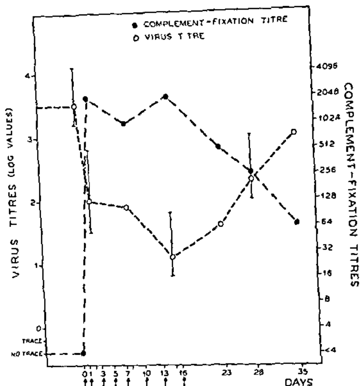


Fig 2

Blood virus titres and complement fixing antibody titres in tolerant virus carrier mice injected eight times with 0 5 ml of serum from immune isologous donors † indicates serum injections

going experiment It was injected into five C3H virus carriers Each mouse was given eight injections, the first five intravenously and the last three intraperitoneally All the injections consisted of 0 5 ml of serum and were given at intervals of 1 3 days throughout a period of 16 days The result is recorded in Fig 2 It will be seen that as long as a high antibody titre was maintained in the virus carrier mice, all showed a virus titre depression in the blood In some the titre reduction was more than 2 log However, this effect did not last Shortly after the serum treatment had been discontinued the titres of the virus in all animals rose to the original level Again, this happened in spite of the presence of quite high antibody titres

Effect of Homologous Lymphoid Cells

It was known from previous experiments that 100 × 10^6 homologous lymphoid cells from immune donors had no effect on the virus in tolerant mice Reinvestigation of the matter confirmed that cell doses of 100 × 10^6 lymphoid cells from immune C3H donors did not influence

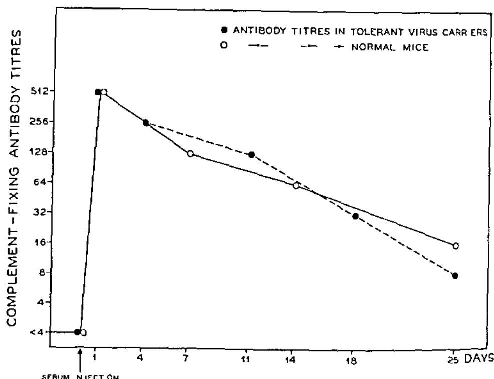


Fig 1

Complement fixing antibodies conferred to normal mice and to tolerant virus carrier mice by one injection intravenously of 0 5 ml of serum from immune isologous donors

after the serum injections tended to be a little lower than the previous experiment More mice among the virus carriers had antibody titres of only ¼ of the titre of the injected serum than was the case when normal animals were employed The number of animals used is too small to decide whether this minor difference between the two groups is apparent or real, but the experiments revealed clearly that also in virus carriers high and rather long lasting antibody titres could be obtained by serum injections

Attempts to eliminate the virus in tolerant mice The effect of one intravenous injection of immune serum was investigated first The serum used had a complement fixation titre of 4096 and a logarithmic neutralization index of 3 5 Five C3H virus carriers received injections One day after the serum injection the mean value of the individual blood virus titres had dropped one log One of the mice had a titre reduction of as much as 1 7 log, but in another no virus titre depression could be detected Judging from the mean values of the individual titres, a modest depression of the virus was detectable for as long as two weeks It then ceased and all titres returned to the original high level This happened in spite of the fact that complement-fixing antibodies were still present in titres of 64–128

In the second experiment much larger quantities of antibodies were employed The serum used was from the same batch as in the fore-

became demonstrable. As in the experiments with 100 × 10^6 homologous cells the immune response was only short-lived. The peak antibody titres were reached within 10 days and from then onwards a rapid decrease was noted, indicating that from the 10th day no antibodies were produced. Nevertheless, during the time the immune response took place a clear cut reduction of the blood virus titres was demonstrable. However, when the serological response ceased, the virus titres returned to the original high level.

As one transplantation dose of homologous cells, if large enough, could cause a temporary depression of the virus titres in tolerant mice, the effect of repeated transplantations was investigated. In attempts to avoid accelerated rejection of the grafts caused by transplantation immunity to the cellular antigens genetically different strains of mice were used as donors. The experiments were accordingly carried out as follows: five C_3H tolerant virus carriers were transplanted intraperitoneally with 1000 × 10^6 lymphoid cells from immunized donors. At intervals of six days this transplantation was followed by four others of equal size, also from immune donors but of other strains. The first donor strain was AKA, the others were not inbred but belonged to strains raised at this institute and kept separate for many years. The results were clear-cut. The first transplantation caused the usual picture: the appearance of antibodies to high titres and a drop in the blood virus titres. However, none of the following transplantations had any effect. The antibody and the virus curves resembled completely those seen when only one transplantation was carried out.

Combined Effect of Homologous Cells and Immune Serum

As the state of tolerance to the virus was not terminated either by means of transplantations with homologous lymphoid cells or by injections of immune serum, a combined treatment was attempted. A group of five AKA tolerant virus carriers was chosen for these experiments. Each of these mice received first a transplant of 1000 × 10^6 lymphoid cells from immune C_3H donors. Eight days later the serum treatment began. The serum originated from AKA mice, had a complement fixation titre of 4096 and a log neutralization index of 3.1. In all eight injections of 0.5 ml were given to each mouse, the first five intravenously at intervals of two days and the last three intraperitoneally three days apart. The transplanted cells caused the usual rise in antibodies and drop in virus titres. The serum injections kept the antibody titres high (C.F. ≧ 512) and the virus titres low (< $10^?$) throughout the period of treatment. However, shortly after the serum injections were discontinued the virus titres rose. In some mice full virus titres were reached as early as one week after the last serum injection and at a time when the complement fixing antibody titres were present in titres of 256.

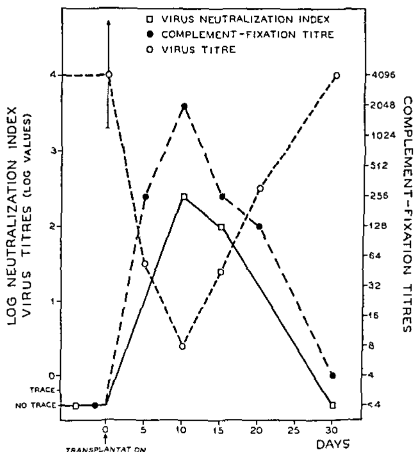


Fig 3

Blood virus titres complement fixing antibody titres and virus neutralizing antibody titres in tolerant virus carrier mice receiving transplants of 1000×10^6 lymphoid cells from immune homologous donors

the virus in AKA virus carriers However, complement-fixing antibodies were produced by the transplanted cells During the first 10 days after the transplantation, a peak titre of 512 was usually obtained (6) As from the 10th day the antibody titres declined and the declination curves were identical with those seen after injection of immune serum, *i e*, probably no antibodies were produced after the 10th day and all the antibodies disappeared within a month Virus neutralizing antibodies were never demonstrated in any mouse given grafts of 100 $\times 10^6$ homologous cells

When the number of transplanted cells was increased to 1000×10^6 homologous *lymphoid* cells, a much stronger *reaction occurred* The results obtained by grafting this quantity of cells from immune C_3H donors to five tolerant AKA virus carriers are recorded in Fig 3 It will be seen that with this large dose of cells the antibody response obtained was almost *as good as in experiments* with 100×10^6 isologous immune cells (8) Not only complement-fixing antibodies occurred in titres of 2048 or even more, but high titre neutralizing antibodies also

results) has demonstrated that cells enclosed in millipore chambers produce quite high complement-fixing antibody titres in recipient virus carriers without any reduction of the virus titres In other experiments (*Hannover Larsen*, unpublished results) in which normal isologous lymphoid cells were employed, no correlation between complement fixing antibodies and virus titres could be found The rôle of the antibodies is, therefore, still obscure One observation points to the possibility that a direct cellular factor is also important for the transplantation effect on the virus *Volkert & Hannover Larsen* (7) have shown that if enough isologous lymphoid cells are transplanted to tolerant virus carriers, it is the organs in which the grafted cells mainly settle, the spleen and the lymph nodes, which can be freed of virus On the other hand, this is not so in organs such as the kidneys, in which only few lymphoid cells will settle The kidneys have never been freed of their virus by adoptive immunization and these organs may contain virus in titres as high as $>10^3$ in transplanted mice in which no trace of virus is left in the spleen and lymph nodes For the present the most logical conclusion from all the experimental results seems to be that antibodies—at least the neutralizing antibodies—as well as cellular factors are important for the transplantation effect on the virus The identity of the cellular factor, however, is unknown It has been assumed that it might be associated with interferon, but this hypothesis has no experimental support On the contrary, attempts to demonstrate interferon in adoptively immunized tolerant mice have failed completely (8)

SUMMARY

LCM complement fixing antibodies conferred by injections of isologous immune serum have a half-life of about four days in both normal and tolerant virus carrier mice

Injections of potent isologous immune serum can reduce temporarily the blood virus titres in tolerant mice but cannot abolish the state of tolerance

In tolerant virus carrier mice large grafts of homologous lymphoid cells from immune donors cause a temporary rise both in complement-fixing and virus neutralizing antibodies During this serological response a temporary reduction of the blood virus titres occurs However, an abolition of tolerance is not achieved

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DISCUSSION

From the experiments described above, it is apparent that injections of large amounts of potent isologous immune serum, grafting of huge numbers of lymphoid cells from immune homologous donors, or perhaps better a combination of these two procedures, can depress the virus in the blood of tolerant virus carriers However, the effect was only temporary and an abolition of the state of tolerance does not seem to be possible by these means Why this is so is not known Perhaps the depression of the virus was only apparent Only the blood titres were determined and the organs might have contained undiminished amounts of virus It might also be that even if the total quantity of virus was reduced, the virus antigenic mass still left might have been above the level necessary for maintenance of tolerance It might also be that the period of time in which the virus was kept down was not long enough It might be due to a combination of the last two possibilities The attempts to overcome such a situation by repeated transplantation of genetically different homologous cells were disappointing This can be explained if it is assumed that the strains of mice used as donors, also had some common transplantation antigens despite their genetic difference If this was the case, the first grafts would have immunized the recipients against the following transplantations and caused an accelerated host versus graft reaction against them In consequence, the cells in the second, third, fourth, and fifth graft were probably killed so quickly that antibodies were not produced in measurable quantities and the virus titres were not affected If completely non-related mice had been used, the results might have been different In practice, however, it does not seem possible to get such mice In spite of the negative results obtained hitherto, one more chance of breaking the tolerance by homografts might still be left By using x-rays prior to the transplantations, it should be possible to enhance and prolong the function of the grafted cells This might be sufficient to cause a good result, and experiments to investigate that possibility are in progress in this laboratory

Together with the results from isografts in tolerant virus carrier mice (8), the serum and homograft experiments give some idea of the relationship between the virus and the antibodies It is clear that in all cases in which complement fixing and neutralizing antibodies are present in high titres the virus titres are depressed Moreover, the antibody and virus curves are mutually reversed These findings indicate strongly that the antibodies are responsible for the virus reduction However, it can also be seen from the experiments that the virus titres may rise and reach high titres even in the presence of large quantities of complement-fixing antibodies, and when isografts are employed the virus titres remain low, even when a great decline in antibody titres has occurred (8) Moreover one of us (*Hannover Larsen*, unpublished

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Acta path et microbiol scandinav 63 181–187, 1965

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INABILITY OF NUCLEIC ACID ANALOGUES TO INHIBIT THE SYNTHESIS OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

By

CHARLES J PFAU[1], IB RODE PEDERSEN and MOGENS VOLKERT

Received 16 vii 64

Recently, work has begun in an attempt to elucidate the structure and composition of lymphocytic choriomeningitis (LCM) virus It was thought that preliminary classification of the virus could be a useful guide for developing purification techniques and interpretation of results Several recent classification schemes (8, 24) have been based on knowledge of the type of nucleic acid within the virus With this in mind we have followed the suggestion of *Salzman* (16) that inhibitors of DNA synthesis might be used to distinguish between DNA and RNA viruses

Selective inhibition of the synthesis of DNA-containing viruses by 5 halodeoxyuridines, structural analogues of thymidine, has been repeatedly demonstrated (2) On the other hand, no chemically-defined RNA virus has been shown to be effected in this way In accord with this view the nucleic acid of vesicular stomatitis virus has been suggested to be RNA since the virus multiplies equally well in the presence or absence of these inhibitors (7, 9) Indirect evidence of the presence of DNA in infectious bovine rhinotracheitis virus has been presented because its replication is markedly inhibited in the presence of these same halogenated pyrimidines (19)

This paper demonstrates the conditions under which two such inhibitors 5 fluoro 2 -deoxyuridine (FUDR) and 5 bromo-2 deoxyuridine (BUDR) interfere with the synthesis of a DNA virus (vaccinia), and show no effect on the synthesis of a virus (vesicular stomatitis) known to be refractory to these compounds It is shown under the same con-

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ditions, that the growth of LCM is not changed by either of these base analogues

MATERIAL AND METHODS

Growth medium for cells and viruses Cells were routinely grown in Eagle's minimum essential medium (4) supplemented with 10 per cent calf serum (E-medium) Inhibitor studies were carried out in solutions containing equal volumes of E medium and the medium of *Sall et al* (15) plus 2 5 per cent calf serum (E S medium)

Viruses Vaccinia was the American Type Culture Collection strain WR (Western Reserve) The CAM assay procedure has already been described (10) The appropriate vesicular stomatitis (VSV) procedures have been outlined previously (22)

The origin assay and general techniques for LCM may be found in past publications from this laboratory (see reference 22) The tissue culture grown virus prior to use in the inhibitor experiments was subjected to low and high speed centrifugation (11) before being resuspended in E S medium

Inhibitors BUDR (lot 731041) was purchased from Calbiochem AG Lucerne Switzerland FUDR (lot 5 0360) was a gift from F Hoffmann La Roche & Co Basle Switzerland

Cell culture L cells strain 929 were obtained from Microbiological Associates Bethesda Md U S A Stocks were grown in rubber stoppered 1 liter Roux bottles in the presence of E medium Cells were dispersed by incubation with 0 2 per cent trypsin (Nutritional Biochemicals Corporation Cleveland Ohio U S A) dissolved in Ca and Mg deficient phosphate buffered saline (3) After one minute the trypsin solution was poured off and incubation was continued for 9 minutes at 37° C. The cells were resuspended in E medium counted and seeded onto 5 cm plastic petri plates (Nunclon m/r type Nunc A/S Roskilde Denmark) The number of cells used was sufficient to produce a monolayer containing between 6 and 8 × 10⁶ cells/plate within 2 to 4 days when incubated at 37° C in a humidified atmosphere of 5 per cent CO_2 in air At this point the plates were used for the inhibitor studies

Virus and cell growth experiments The monolayers as described above were exposed to virus (the specific details may be found in the appropriate figures) for 1 hour at 37° C After this incubation period the solutions were removed (5 ml E-S medium/plate) and the monolayers washed several times with E S medium The amount of cell adsorbed virus was determined (in most of the experiments) by assaying one of these washed monolayers after it had been frozen and thawed three times Duplicate plates were then filled with E S medium and the appropriate amounts of inhibitor (as shown in the figures) In certain experiments the number of LCM infected or non infected cells/plate in the presence or absence of inhibitors were determined at daily intervals The supernatants were removed (and assayed if infected cultures were used) and the monolayers dispersed by trypsinization (as above) The cells were resuspended and counted and if infected frozen and thawed three times before being assayed It has been shown previously that trypsin has no effect on LCM infectivity (11) and that the tissue culture fluids can be frozen and thawed up to 15 times without loss of infectious virus (12)

RESULTS

L cell multiplication in the presence of BUDR and FUDR Kjellen (6) found that unlike FUDR, the concentration of BUDR necessary to prevent MAS A cell proliferation was equal to that necessary to inhibit formation of infectious adenovirus In the event that such conditions were operative in our system experiments were performed to establish the BUDR concentration necessary to inhibit L cell proliferation Monolayers containing 5 × 10 cells were exposed to BUDR at various concentrations (0, 2 20, and 40 µg/ml) Each day for the next four days 4 of the appropriate monolayers were dispersed by trypsinization Cell

counts showed essentially the same results as found by *Kjellen* Two days after exposure to 20 and 40 μg ml of BUDR cell multiplication had almost ceased while at the 2 μg ml level it was identical to the control cultures Similar experiments using FUDR showed, however, that this compound at even 2 μg ml began to inhibit cell proliferation within 24 hours These latter results are similar to those found by *Simon* (17) when using HeLa cells

Vaccinia virus synthesis in the presence of BUDR and FUDR The efficiency of these analogues to inhibit a known DNA virus (10) was checked before beginning the LCM experiments As shown in Table 1, when these compounds were incorporated into the test system the yield of infectious virus was reduced 1000 to 10000 fold

TABLE 1

Inhibition of Vaccinia Virus Synthesis by 5 halodeoxyuridines Inhibitor added at the Time of Infection

Analogue	Conc (μg ml)	Virus titre (PFU ml)
BUDR	0	$6\,5 \times 10$
	20	$1\,3 \times 10^5$
	80	$4\,2 \times 10^3$
FUDR	0	$5\,6 \times 10^7$
	2	$1\,2 \times 10^4$
	20	$1\,7 \times 10^4$

2×10^6 PFU (pock forming units) of vaccinia virus (in 5 ml volumes) were placed on each plate at the beginning of the adsorption period After one hour the amount of cell adsorbed virus was $3\,9 \times 10^3$ (in the FUDR experiment) Total harvests (supernatants + disrupted cells) were made 48 and 42 hours later for the BUDR and FUDR experiments respectively

TABLE 2

VSV Synthesis in the Presence of FUDR Inhibitor added at the Time of Infection

FUDR conc (μg ml)	Virus titre (PFU ml) $\times 10^8$
0	1 1
2	1 0
20	1 6

10^3 PFU (plaque forming units) of VSV (in 5 ml volumes) were added to each plate at the beginning of the adsorption period Total harvests (supernatants + disrupted cells) were made 48 hours later This data is the average of two experiments in which the virus inoculum was identical

VSV synthesis in the presence of FUDR In order to check the specificity of FUDR as used in the above experiment VSV was grown under the same conditions As can be seen in Table 2 no such inhibition of virus synthesis occurred

LCM synthesis in the presence of BUDR and FUDR Tables 3 and 4 show the infectivities of LCM cultures in the presence and absence of BUDR and FUDR respectively In both types of experiment the inhi

bitors were added after the virus adsorption period In one experiment (Table 5) the virus was grown in cells exposed to FUDR 48 hours prior to the time of infection All the results show that there is no great effect (an average of 40 per cent drop in titre) on LCM synthesis in the presence of these analogues

TABLE 3

LCM Synthesis in the Presence of BUDR Inhibitor added at the Time of Infection

BUDR conc (µg ml)	I D₅₀ ml (time after infection)		
	48 hrs $\times 10^{6}$	72 hrs $\times 10^{6}$	96 hrs $\times 10^{6}$
0	3 3	1 1	0 19
20	1 1	0 33	1 9
80	1 9	1 1	0 11

1 1 $\times$ 10⁶ LD_{50} units of LCM (in one ml volumes) were placed on each plate at the beginning of the adsorption period After one hour the amount of cell adsorbed virus was 1 6 $\times$ 10⁴ The media plus the disrupted monolayers were frozen and thawed three times before assaying

TABLE 4

LCM Synthesis in the Presence of FUDR Inhibitor added at the Time of Infection

FUDR conc (µg ml)	Part analysed	I D₅₀ ml (time after infection)		
		24 hrs $\times 10^{6}$	48 hrs $\times 10^{6}$	72 hrs $\times 10^{6}$
0	medium	4 8	20	6 0
	cells	–	2 4	0 46
2	medium	2 5	6 0	6 0
	cells	–	–	–
20	medium	2 6	8 9	3 9
	cells	–	0 73	0 046
80	medium	2 5	1 9	6 0
	cells	–	0 46	0 046

6 3 $\times$ 10⁶ LD_{50} units of LCM (in 1 ml volumes) were placed on each plate at the beginning of the adsorption period After one hour the amount of cell adsorbed virus was 5 9 $\times$ 10⁴ ID_{50} units/plate The culture fluid and the resuspended trypsin dispersed monolayers were assayed independently This data is the average of two experiments in which the virus inoculum was identical

TABLE 5

LCM Synthesis in the Presence of FUDR Inhibitor added 48 Hours Prior to the Time of Infection

FUDR conc (µg ml)	I D₅₀ ml $\times$ 10
0	5 9
2	3 3
20	3 3

Duplicate petri plates containing about 6 $\times$ 10⁵ cells plate were exposed for two days to media containing either 2 or 20 µg/ml of FUDR The plates not exposed to inhibitor contained 2 $\times$ 10⁵ cells so that at the time of virus infection all plates contained about the same number of cells The virus inoculum and adsorption period were the same as presented in Table 4 48 hours after infection the media and monolayers were frozen and thawed three times before assaying

DISCUSSION

From a review of the current literature it seems reasonable to assume that a virus does not contain DNA if its synthesis is not inhibited by BUDR and FUDR This of course, must be done in a system in which the specificity of these analogues has been demonstrated Since the mode of action of these two inhibitors is dissimilar, use of both independently acts as a further check on their specificity The results of our test system have confirmed the already well established fact that vaccinia virus synthesis is inhibited by FUDR (16) and BUDR (5) Moreover VSV, as expected was not inhibited under the same conditions

As previously reported (23), L cells infected with LCM normally show no cytopathic changes The present study has also confirmed earlier observations that the number of cells in virus infected cultures increase at about the same rate as their non-infected counterparts Similarly, proliferation of cells in virus infected cultures is stopped by base analogues In the BUDR experiments (Table 3), 48 hours after infection, there were nearly twice as many cells in the inhibitor free cultures as in the ones exposed to this analogue At 72 hours there were three times as many, and after that cell proliferation ceased—being about 5–6 × 10^6 or the maximum number of cells per plate The same was true in the FUDR experiments (Table 4) except that cell multiplication in the presence of this inhibitor almost completely ceased after 24 hours

The small decrease in infectious LCM titre observed in two thirds of the inhibitor treated cultures (Tables 3 and 4) cannot be compared to the almost virtual elimination of synthesis of viruses of the DNA type Furthermore, it is independent of the concentration of the inhibitor used In one experiment (not reported here) the concentration of BUDR was varied from 2 mg ml to 2 µg/ml with no observed difference in the virus titres produced in the various concentrations of inhibitor Preliminary experiments (13) of the same type show that actinomycin D has no effect on the synthesis of LCM in L cells This again suggests that LCM does not contain DNA (14)

The data presented in Tables 3 and 4 also indicate that the inhibitors do not interfere with the growth rate of the virus Both in treated and non treated cultures the maximum titre of LCM was reached on the [illegible]

[illegible] Table 4 this re- [illegible]

[illegible] re readily inac- [illegible] in the presence rather than in the absence of Mg^{++} and Ca^{++} (11) That LCM is ether sensitive (1, 20) has been confirmed (11) If it is

assumed now that LCM contains RNA, according to the scheme of *Wallis & Melnick* (24) it would fit into the arbor or myxo group of viruses. It has not been serologically related with these or other groups of viruses (18). Like arbor viruses LCM is sensitive to deoxycholic acid (12, 21), but this is also a property of influenza A (21). The best criteria at the moment, for distinguishing which of these groups LCM might belong to, seem to be size and structure. This area is where little is known about the virus. Its size would seem to lie between the ranges usually given for these groups being closer to the myxo than to the arbor viruses. Current investigations are aimed at elucidating its architecture, as well as chemically establishing the nature of its nucleic acid.

SUMMARY

Evidence is presented which suggests that LCM virus does not contain DNA. *This is based on the inability of FUDR and BUDR to suppress* the synthesis of the virus. Under identical experimental conditions these base analogues have been shown to block the synthesis of a DNA virus (vaccinia), and cause no change in the production of a virus thought to contain RNA (VSV). Classification of the virus, based on its presumed nucleic acid content as well as other properties, is discussed.

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The Rickettsia & Virus Department Statens Seruminstitut, Copenhagen Denmark

BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

1 *Density Gradient Studies*

By

CHARLES J PFAU[1]

Received 22 viii 64

Lymphocytic choriomeningitis (LCM) virus is well known for its ability to produce a carrier state in mice, yet very little is known about the virus itself Ultrafiltration and centrifugation analyses have indicated its probable size to be between 37 and 60 mμ (16) That this agent is not subviral (infectious nucleic acid), is supported by its sedimentation properties (17, 21) as well as its resistance to nucleases (21) Sensitivities of virus preparations to heat (2, 10), pH (1, 2), soaps (19) and ether (4, 19) have also been reported

Studies have been initiated in this laboratory to learn more about the nucleic acid content (13), structure, and replication of this virus This paper is concerned with structural, or more specifically, purification studies on LCM

The use of density gradient centrifugation for the isolation and purification of viruses has been one of the major developments in biochemical virology In essence the technique involves the separation of particles partially or entirely on the basis of their buoyant density in a convection free medium If a virus has a buoyant density sufficiently different from that of contaminating particles, and if the salts used to form the gradient are not harmful to the virus, purification and quantitative recovery of the virus may (in most cases) be achieved Use of this technique as a possible way of purifying LCM has now

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I am indebted to Dr *Preben von Magnus* Director of the Statens Seruminstitut for making my stay here possible None of the experiments reported here could have [illegible] the whole hearted cooperation of the chief of [illegible] *Volkert* It is indeed a pleasure to [illegible] nd his many helpful suggestions in [illegible] from useful discussions with Mag [illegible] *arsen* Various parts of this invest [illegible] tance of Miss *L Christiansen* Miss [illegible]

been considered The results of preliminary studies on the behavior of the virus in density gradients are the subject of this report

MATERIAL AND METHODS

Virus and Assay Techniques

The strain of LCM was that described in previous publications from this laboratory Titration methods have already been outlined (20) and unless otherwise stated the infectivities are expressed as the log of the LD_{50}/ml (with the decimal part of the power function in whole numbers) The term "Total Infectivity" is the product of the LD_{50}/ml and the number of ml (or the fraction thereof) used in a defined experiment

Tissue Culture Techniques

Using several cell lines we have grown the virus under a variety of conditions

thawed just before use

Density Gradient Techniques

Unless specifically described the methods have already been summarized (12) All salts except where noted were dissolved in pH 7 4 phosphate buffered saline (PBS) without added Ca^{++} or Mg^{++} (8) The following four types of gradients were used

[illegible] For the density gradient part of the experiment the virus solution was adjusted to a density of 1 26 and was spun in the SW 39 rotor for 18 hours at 35 000 RPM

[illegible] method II' [illegible] ng a density [illegible] except that

[illegible] hours Fractionation w[illegible] lecting 12 drop sample[illegible] since the concentratio[illegible] the mice

4 *Sucrose* As in 3 except that 20–5 per cent preformed sucrose gradients were used

Thermal Inactivation Techniques

These were carried out essentially as described by Wallis *et al* (23) as the "heating methods"

Enzyme Techniques

These methods have already been described for LCM (21)

Concentration and Precipitation Techniques

1 *Carbowax 4000 and 6000* The procedure for concentrating virus solutions by the use of these saturated polyethylene glycols has been described previously (12)

2 *Protamine sulfate* To each 10 ml of tissue culture fluid was added 100 mg of protamine sulfate (suspended in 2 ml of PBS at room temperature) After 30 minutes of shaking at 4° C the solution was centrifuged lightly and the pellet resuspended with PBS

3 *Alcohols and ammonium sulfate* All the following procedures were carried out at 4° C To 15 ml aliquots of LCM tissue culture fluid, adjusted to pH 7 0 with dilute NaOH sufficient quantities of either methanol, ethanol or ammonium sul phate solution (PBS saturated at 4° C) were added to produce colloidal looking solutions They were then spun for five minutes at 1000 × G and the supernatants as well as the pellets (resuspended in 10 ml of PBS) were assayed The entire pro cedure was repeated with the resuspended pellets

Chemicals

The sources of chemicals used (all of the highest purity obtainable) were as fol lows protamine sulphate from the British Drug House Ltd , RbCl from American Potash and Chemical Co , CsCl (optically pure grade) from Harshaw Chemical Co potassium tartrate from Allied Chemical Co , sucrose (preparation for microscopy and bacteriology) from E Merck AG, DNase and RNase from Worthington Biochemical Corp , trypsin from L Light & Co , pronase from Calbiochem AG, Carbowax 4000 and 6000 as well as Visking cellulose tubing, from Union Carbide Chemicals Co Methanol, ethanol $CaCl_2$ and $MgCl_2$ were analytical grade reagents

EXPERIMENTAL

Preparation of Virus Suspensions Prior to Density Gradient Centrifugation

Initially, efforts were made to obtain solutions containing the highest possible virus titres prior to their use in density gradient experiments The following precipitation and concentration techniques were used

1 *Protamine sulphate* Virus could be quantitatively recovered from precipitates only if they were resuspended in the original volume of fluid used Since resuspension in smaller volumes led to large apparent losses in infectivity this procedure could not be used for concentration purposes

TABLE 1

Effect of Precipitating Agents on LCM Infectivity

No	Material	Fraction assayed*	ID_{50} ml 10^5	Volume (ml)	Total infectivity × 10^6	% recovery
1	Tissue culture fluid		33	15	50	–
2	1 + 5 2 ml	S	3 3	18 1	5 9	12
3	of methanol	P	59	10	59	118
4	2 + 4 5 ml	S	<1 1	20 1	<2 1	<35
5	of methanol	P	3 3	10	3 3	56
6	1 + 7 1 ml	S	<1 1	19 7	<2 1	<4 2
7	of ethanol	P	<1 1	10	<1 1	<2 1
8	1 + 6 0 ml	S	5 9	18 6	11	22
9	of $(NH_4)_2SO_4$	P	3 3	10	3 3	6 7

* After centrifugation the supernatants (S) were decanted and the pellets (P) re [illegible]

re not given past
was not achieved

2 *Alcohols and ammonium sulphate* As seen in Table 1, only Methanol could be used to quantitatively precipitate LCM As in the pro tamine sulphate experiments resuspension of precipitates in volumes

considerably smaller than those of the original led to large decreases in total infectivities

3 *Carbowax 400 and 6000* When either of these saturated polyethylene glycols were used to reduce the volume of LCM tissue culture fluid by a factor of ten (*usually accomplished in 3 to 4 hours at 4° C*), less than 5 per cent of the original infectivity remained Control experiments showed that virus solutions surrounded by either Visking tubing or glass lost little infectivity during that time interval Various concentrations of these carbowaxes were added to non concentrated tissue culture fluids in an attempt to demonstrate their possible toxic effect but no such effect could be demonstrated

4 *Sedimentation onto high density salt cushions* As seen in Table 2 after centrifugation of tissue culture fluid 77 per cent of the virus could be recovered from the liquid at the interface between the tissue culture fluid and the RbCl cushion The virus recovered in this way was suspended in about 5 per cent of the original volume of tissue culture fluid used

TABLE 2

Infectivity of LCM Virus in Rubidium Chloride Density Gradient Experiments

Sample	Material	ID_{50} ml $\times 10^6$	Volume (ml)	Total infectivity 10^6	% original infectivity
1	Tissue culture fluid	18 8	70	1310	
2	Interface fluid	333	3 0	1000	77
3	Sample 2 dilut 1/100 + 18 hrs 4° C	0 59	2 0	177	13 6
4	Gradient tube 1 non-centrifuged 18 hrs 4° C	187	5 0	935	71
5	Gradient tube 2 centrifuged and mixed	1 05	5 0	5 25	0 4
6	Gradient tube 3 tube 1	0 33	0 4	0 133	0 013
7	Gradient tube 3 1 and 2	<0 0105	0 4	<0 0042	<0 00032

The
at

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(sa
The latter was used to fill 3 S
th (sample 4) placed at 4° C
tubes was inverted mixed and
was punctured and the two [illegible] and assayed (samples 6 and 7)

Density Gradient Experiments

1 *RbCl* The results of a typical experiment are presented in Table 2 As stated above sample 2 shows that most of the virus was recovered

from the interface This interface material, suspended in 10 5 M RbCl (sample 4), lost much less of its infectivity after standing for 18 hours at 4° C than the same material diluted 100-fold in PBS (sample 3) before the beginning of incubation Yet virus in this high ionic strength medium lost close to 99 5 per cent of its infectivity after centrifugation for the same period (sample 5 vs 2) Furthermore the infectivity of the isolated bands (samples 6 and 7) was only 2 6 per cent of that found by inverting and assaying the other centrifuged tube (sample 5) containing the identical material

2 *CsCl* The results of these experiments are essentially in agreement with those found when using RbCl Again the infectivity of the material in the inverted centrifuged tubes was only between 1 and 10 per cent of their non-centrifuged counterparts As with RbCl, virus suspended in CsCl was inactivated less than virus in lower ionic strength media

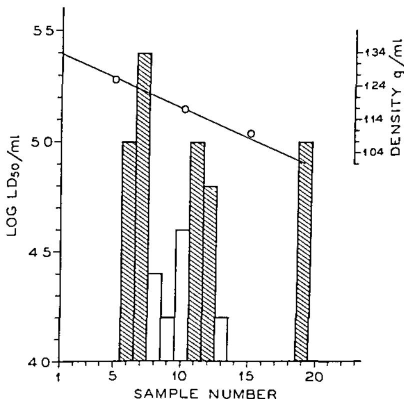


Fig 1

Equilibrium sedimentation of LCM virus using preformed potassium tartrate density gradients Solid tartrate was added to two tissue culture samples to give 60 and 30 per cent concentrations respectively and were assayed after standing at 4° C for two hours (Table 3 No 2 and 3) One of the two centrifuged samples was inverted mixed and its contents assayed (Table 3 No 4) while the other tube was fractionated A non infectious band was collected in sample 5 while the next two bands 6–7 and 11 12 (darkened in the figure) were infectious The densities of these infectious bands were 1 24 and 1 15 respectively Infectious fluid was also associated with the fluid between these two bands (samples 8–10) Sample 19 (darkened in the figure) represents the infectivity associated with the gradient tissue culture interface

TABLE 3

Recovery of Infectious LCM Virus after Potassium Tartrate Density Gradient Centrifugation

No	Material	Total infectivity × 10⁶	% original infectivity
1	Non centrifuged tissue culture fluid	1 47	–
2	1 in 60 % tartrate	1 23	83
3	1 in 30 % tartrate	1 37	93
4	Gradient tube 1* inverted and mixed	0 135	9 2
5	Gradient tube 2* fractionation samples 6–13 and 19	0 141	9 6

* See Fig 1 for more details of this experiment

3 *Potassium tartrate* The results of a typical experiment using this type of gradient are presented in Table 3 and Fig 1 Besides the two sharp bands which were visible in the RbCl and CsCl experiments, a small non-infectious band (sample 5) was apparent immediately below the more dense of the two major bands Again, over 90 per cent of the infectivity was lost (4 vs 1 in Table 3), while non-centrifuged virus in different tartrate concentrations showed little loss in infectivity (2 and 3 in Table 3) The total infectivity of the 24 collected fractions agreed quite well with that determined by inverting and mixing (4 vs 5 in Table 3) In other experiments in this series, each of the three bands was removed individually and, after adjusting their volume to 1 ml with PBS, placed over 50 10 per cent tartrate gradients After spinning under the above conditions the relative positions of the bands did not change, but none were infectious (< LD_{50} 2 5)

4 *Sucrose* As in the Rb and CsCl experiments, two bands were visible and less than 10 per cent of the original infectivity was found after centrifugation Control experiments showed that the concentration of sucrose used was not toxic to the virus

Stabilization Experiments

Crawford (7) had found that full recovery of Rous Sarcoma could be achieved only if the RbCl was dissolved in sodium citrate buffer and 0 02 per cent plasma albumin In our cesium chloride experiment the salt [illegible]

[illegible] of the gradients was adjusted to 6 0, 7 0, 7 5, or 8 0 without any indication of better recovery of virus *Melnick* and his co workers (22, 23) have found that certain viruses are almost completely stabilized against thermal inactivation by high concentrations of mono- and divalent cations The stability of LCM in the presence of Na, Mg^{++}, and Ca^{++} was determined with the thought that, if the virus was stabilized, these cations might exert the same protective effect when incorporated into the density gradients As can be seen in

Table 4, LCM clearly falls into the class of viruses which are much more labile to thermal inactivation in the presence, rather than in the absence, of di-valent cations. Na^+, while not appreciably increasing thermal inactivation definitely shows no protective effect.

TABLE 4

Thermal Inactivation of LCM in the Presence of Different Cations

Final cationic concentration	LD_{50} non purified virus temp (°C) time (min)		LD_{50} semi purified virus temp (°C) time (min)
	37 85	50 12	37 15
Control	3.5	3.0	3.0
2 M NaCl	2.5	–	2.75
1 M NaCl	3.5	–	3.0
1 M $MgCl_2$	<0.5	<0.5	<0.5
1 M $CaCl_2$	<0.5	<0.5	<0.5
Eagle's medium	3.5	–	3.0

The results presented here were obtained with the same lot of tissue culture fluid either before or after differential centrifugation (14). All fluids were diluted 50 fold in PBS or (in one case) Eagle's medium. The times of incubation at either 37 or 50° C were such that at least one log unit of infectivity was lost when compared to the control at 4° C. The initial log LD_{50} of the tissue culture fluid as well as the semi purified virus was 4.5.

In the Rb and CsCl density gradient experiments it was observed that virus suspended, but not centrifuged, in these high salt concentrations lost little of its activity after 18 hours at 4° C. However, it has been our experience that most of the infectivity (> 90 per cent) would have been lost in the same tissue culture fluids (without added salt) after that incubation period. Since many enzymes are inhibited in high ionic strength solutions, it seemed that such an explanation could be used for the stabilization effect. In summary, no decrease in virus infectivity could be detected after either DNase or RNase treatment. The same results were obtained after incubation with either trypsin of pronase (1 mg/ml) for 1 hour at 37° C.

DISCUSSION

Density gradient techniques, when applied to LCM virus, have invariably led to loss of 90 per cent of the infectious virus even though the Rb, Cs, and tartrate salts *per se* were harmless to the virus (Tables 2 and 3). The same effect was found in sucrose gradients even though sucrose (under certain conditions) appears to reverse aggregation of virus particles (14). This seems to eliminate one of the possible factors which could be responsible for loss of infectivity after gradient centrifugation. Two other obvious possibilities to account for this loss are separation of some non-viral stabilizing component from the virus, or damage (or loss) of a vital part of the virus during

gradient centrifugation With respect to the former possibility, whatever this theoretical substance might be, its dissociation from the virus is not reversible This seems to be so, since the total infectivity of the fractionated gradients is identical to the same preparation which had been thoroughly mixed before being assayed (Table 3) With respect to the latter possibility, it is conceivable that LCM might possess an enveloped capsid (the terminology of different viral subunits is that used by *Lwoff et al*, ref 11) Although a general statement about the necessity of envelopes for full expression of viral infectivity cannot be made, *Smith* (18) has recently found that it does seem to be quite important for herpes simplex virus[1] Thus it does not seem illogical to suggest that some sort of damage to LCM envelopes, upon density gradient centrifugation, has led to loss of most of the infectious virus A similar type of argument has been proposed recently by *Plus* (15) to account for losses of infectivity when subjecting sigma virus to sucrose density gradient centrifugation

The potassium tartrate fractionation experiments (Fig 1) have shown that LCM infectivity is associated with the two distinctly visible bands which were formed during density gradient centrifugation These bands were visible even after the virus had been partially pu-

in a virus population (see reference 12) However, since this was not so, it might just as easily represent adsorption of the virus to different types of contaminating particles This is probably the case, since very weak bands in the same positions were observed when non infected tissue culture fluids were used However, the same phenomenon is observed with many other virus systems

Compared to the infectivity associated with the tartrate bands (Fig 1 samples 6–7 and 11–12), there was a seemingly large amount of infectivity left at the tissue culture—density gradient interface (sample 19) This was so even when fractions were collected using a top-unloader (12) to avoid contamination by the lower infectious layers This amount of virus may be considered small, however, if one considers that the infectivity of the virus that has *just reached* the gradient is being compared with that which has *passed into* it The experiment with RbCl (Table 2) showed that most of the virus was recovered after it had sedimented onto the surface of a RbCl solution, but not after it had passed into the same less dense solution Thus, most of the infectivity is lost by passage of the virus into and through the gradient, while virus reaching the surface, and no further, is not damaged The densities of the infectious material in the tartrate bands (1 15 and

[1] It has been shown that herpes simplex is destroyed by a phosphatase (3) which can be inhibited by lysine Using *Eagles* medium (9) which contains lysine, LCM could not be protected against thermal inactivation (Table 1)

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2 M NaCl	2 5	–	2 75
1 M NaCl	3 5	–	3 0
1 M $MgCl_2$	<0 5	<0 5	<0 5
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The potassium tartrate fractionation experiments (Fig. 1) have shown that LCM infectivity is associated with the two distinctly visible bands which were formed during density gradient centrifugation. These bands were visible even after the virus had been partially purified by either differential centrifugation (14) or methanol precipitation (Table 1). If a purified virus preparation were used, the idea could be entertained that this might represent different classes of particles in a virus population (see reference 12). However, since this was not so, it might just as easily represent adsorption of the virus to different types of contaminating particles. This is probably the case since very weak bands in the same positions were observed when non-infected tissue culture fluids were used. However, the same phenomenon is observed with many other virus systems.

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1 M CaCl	<0.5	<0.5	<0.5
Eagle s medium	3.5	–	3.0

The results presented here were obtained with the same lot of tissue culture fluid either before or after differential centrifugation (14) All fluids were diluted 50 fold in PBS or ... 37 or 50° C
were such
trol at 4°
virus was 4 5

In the Rb and CsCl density gradient experiments it was observed that virus suspended, but not centrifuged, in these high salt concentrations lost little of its activity after 18 hours at 4° C However, it has been our experience that most of the infectivity (> 90 per cent) would have been lost in the same tissue culture fluids (without added salt) after that incubation period Since many enzymes are inhibited in high ionic strength solutions, it seemed that such an explanation could be used for the stabilization effect In summary, no decrease in virus infectivity could be detected after either DNase or RNase treatment The same results were obtained after incubation with either trypsin or pronase (1 mg/ml) for 1 hour at 37° C

DISCUSSION

Density gradient techniques, when applied to LCM virus, have invariably led to loss of 90 per cent of the infectious virus, even though the Rb, Cs, and tartrate salts *per se* were harmless to the virus (Tables 2 and 3) The same effect was found in sucrose gradients, even though sucrose (under certain conditions) appears to reverse aggregation of virus particles (14) This seems to eliminate one of the possible factors which could be responsible for loss of infectivity after gradient centrifugation Two other obvious possibilities to account for this loss are separation of some non-viral stabilizing component from the virus, or damage (or loss) of a vital part of the virus during

gradient centrifugation With respect to the former possibility, whatever this theoretical substance might be, its dissociation from the virus is not reversible This seems to be so, since the total infectivity of the fractionated gradients is identical to the same preparation which had been thoroughly mixed before being assayed (Table 3) With respect to the latter possibility, it is conceivable that LCM might possess an enveloped capsid (the terminology of different viral subunits is that used by *Lwoff et al*, ref 11) Although a general statement about the necessity of envelopes for full expression of viral infectivity cannot be made, *Smith* (18) has recently found that it does seem to be quite important for herpes simplex virus [1] Thus it does not seem illogical to suggest that some sort of damage to LCM envelopes, upon density gradient centrifugation, has led to loss of most of the infectious virus A similar type of argument has been proposed recently by *Plus* (15) to account for losses of infectivity when subjecting sigma virus to sucrose density gradient centrifugation

The potassium tartrate fractionation experiments (Fig 1) have shown that LCM infectivity is associated with the two distinctly visible bands which were formed during density gradient centrifugation These bands were visible even after the virus had been partially purified by either differential centrifugation (14) or methanol precipitation (Table 1) If a purified virus preparation were used, the idea could be entertained that this might represent different classes of particles in a virus population (see reference 12) However, since this was not so, it might just as easily represent adsorption of the virus to different types of contaminating particles This is probably the case, since very weak bands in the same positions were observed when non-infected tissue culture fluids were used However, the same phenomenon is observed with many other virus systems

Compared to the infectivity associated with the tartrate bands (Fig 1, samples 6–7 and 11–12), there was a seemingly large amount of infectivity left at the tissue culture—density gradient interface (sample 19) This was so even when fractions were collected using a top-unloader (12) to avoid contamination by the lower infectious layers This amount of virus may be considered small, however, if one considers that the infectivity of the virus that has *just reached* the gradient is being compared with that which has *passed into* it The experiment with RbCl (Table 2) showed that most of the virus was recovered after it had sedimented onto the surface of a RbCl solution, but not after it had passed into the same less dense solution Thus, most of the infectivity is lost by passage of the virus into and through the gradient, while virus reaching the surface, and no further, is not damaged The densities of the infectious material in the tartrate bands (1 15 and

[1] It has been shown that herpes simplex is destroyed by a phosph[illegible] can be inhibited by lysine Using *Eagles* medium (9) which [illegible] could not be protected against thermal inactivation (Table 1)

Table 4, LCM clearly falls into the class of viruses which are much more labile to thermal inactivation in the presence, rather than in the absence, of di-valent cations Na^{+}, while not appreciably increasing thermal inactivation definitely shows no protective effect

TABLE 4

Thermal Inactivation of LCM in the Presence of Different Cations

Final cationic concentration	LD_{50} non purified virus temp (°C) time (min)		LD_{50} semi purified virus temp (°C) time (min)
	3 85	50 12	3 15
Control	3.5	3.0	3.0
2 M NaCl	2.5	–	2.75
1 M NaCl	3.5	–	3.0
1 M $MgCl_2$	<0.5	<0.5	<0.5
1 M $CaCl_2$	<0.5	<0.5	<0.5
Eagle's medium	3.5	–	3.0

The results presented here were obtain
either before or after differential centrifi
in PBS or (in one case) Eagle's medium
were such that at least one log unit of infectivity was lost when compared to the control at 4° C. The initial log LD_{50} of the tissue culture fluid, as well as the semi purified virus was 4.5

In the Rb and CsCl density gradient experiments it was observed that virus suspended, but not centrifuged, in these high salt concentrations lost little of its activity after 18 hours at 4° C However, it has been our experience that most of the infectivity (> 90 per cent) would have been lost in the same tissue culture fluids (without added salt) after that incubation period Since many enzymes are inhibited in high ionic strength solutions, it seemed that such an explanation could be used for the stabilization effect In summary, no decrease in virus infectivity could be detected after either DNase or RNase treatment The same results were obtained after incubation with either *trypsin* of pronase (1 mg/ml) for 1 hour at 37° C

DISCUSSION

Density gradient techniques, when applied to LCM virus, have invariably led to loss of 90 per cent of the infectious virus, even though the Rb, Cs, and tartrate salts *per se* were harmless to the virus (Tables 2 and 3) The same effect was found in sucrose gradients, even though sucrose (under certain conditions) appears to reverse aggregation of virus particles (14) *This seems to eliminate one of the possible* factors which could be responsible for loss of infectivity after gradient centrifugation Two other obvious possibilities to account for this loss are separation of some non viral stabilizing component from the virus, or damage (or loss) of a vital part of the virus during

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1 24) do not contradict the earlier assumption that the density of the virus was 1 2 *McNair Scott & Elford* assumed this density (16) when calculating the size of LCM from ultracentrifugation data As the authors stated "Should the density of this virus differ from 1 2 by ± 0 05 per cent, then a correction amounting to —10 or +15 per cent respectively would need to be applied to the calculated particle diameter"

Some comment should also be made about the experiments involving precipitation of LCM virus with methanol, ethanol, and ammonium sulphate Perhaps under different conditions of pH, temperature, or alcohol concentration, good results with ethanol might have been obtained Methanol is the alcohol of choice when precipitating influenza virus As pointed out by *Cox* and his co-workers (6), the concentration range for quantitative recovery of influenza was much broader with methanol than ethanol They felt that methanol produced less denaturation of protein than ethanol Turning now to our results with ammonium sulphate, it is even more difficult to explain its deleterious influence on the virus This has been considered a very good way to precipitate proteins and viruses, but in this case the ion may be in the same class as Ca^{++} and Mg^{++} (Table 4)

SUMMARY

It has not been possible to quantitatively recover LCM virus after centrifugation in self establishing density gradients of RbCl or CsCl, as well as in preformed gradients of potassium tartrate and sucrose This loss (over 90 per cent of the original infectivity) is irreversible and independent of hydrogen ion, citrate, or serum concentration in the gradient The infectious virus that is recovered from the gradients is associated with two bands having densities of 1 15 and 1 24 This density heterogeneity does not appear to be an artifact of the technique used Reasons for this dispersion, as well as the instability of the virus, are discussed

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demonstrate the rôle of the above factor in the recovery of sedimented infectious virus It will be shown that use of solutions which reverse aggregation also enables the virus to withstand fluorocarbon treatment —another commonly used purification procedure

MATERIAL AND METHODS

Centrifugation All sedimentation procedures were performed in the Spinco model L preparative ultracentrifuge Rotor temperatures were maintained between 3 and 5° C Minimum apparent sedimentation rates were calculated according to the method of *Trautman* (13)

Ether sensitivity tests These were carried out according to the technique described by *Andrews & Horstmann* (1)

for 30 seconds at l w speed Separation of the two phases was accomplished by centrifugation at 1000 × G for 5 minutes Then the aqueous phase was treated with 1 ml of F114 [illegible]

EXPERIMENTAL

Centrifugation In an attempt to duplicate the differential centrifugation conditions used by *Smadel* (11), as well as keep our own consistent the system proposed by *Trautman* (13) has been employed From the data in the *Bauer & Pickels* paper (2), we have estimated the ST (13) of their rotor to be 167 Combined with the published data (11) it appears that centrifugation sufficient to pellet components with minimum apparent S rates (S_{min}) greater than 15 000 did not bring down infectious material[1] Using the Spinco No 40 rotor, various times and speeds were used to determine the highest S rates which would sediment less than 10 per cent of the infectious virus in the tissue culture fluid used by us This was found to be 10 000 RPM for 15 minutes giving an S_{min} of 4840 (Table 1) By the same type of procedure conditions were established so that less than 10 per cent of the infectious virus remained in the supernatants Under these conditions particles having S rates greater than 178 S (*Table 1*) were pelleted

Resuspension of sedimented virus Using the conditions established above for quantitatively sedimenting FCM virus from tissue culture fluids various solutions were used to resuspend the pelleted virus Re-

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BIOPHYSICAL AND BIOCHEMICAL CHARACTERIZATION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

2 *Partial Purification by Differential Centrifugation and Fluorocarbon Techniques*

By

CHARLES J PFAU[1]

Received 27 viii 64

In a previous communication (9) studies on the behaviour of LCM virus in density gradients were presented Because of the consistent loss of over 90 per cent of the infectious virus after this type of centrifugation, other techniques for purifying the virus were considered

Differential centrifugation, a well known way for purifying viruses, has been employed by *Smadel* and his coworkers (11) to concentrate LCM virus They found that when a 10 per cent suspension of infected splenic tissue was centrifuged at 10,000 RPM for 6 to 10 minutes in the original *Bauer & Pickels'* type of rotor (2), little infectivity was associated with the sediment When the supernatant was spun at 30,000 RPM for 20 minutes or 20,000 RPM for 30 minutes, most of the virus was pelleted A crucial factor at this stage was the type of medium used to resuspend the pellet Among a variety of diluents employed, only physiological saline plus 2 per cent normal inactivated guinea pig serum would allow full expression of the infectious virus in the resuspended pellet This finding has been confirmed by us with the exception that calf serum was used instead of guinea pig serum The need to eliminate a serum-containing diluent in any purification scheme is obvious

It seemed possible that serum might be reversing aggregates formed on sedimentation of the virus Such aggregation is common in several virus systems and, in many instances, can be reversed by use of either low or high ionic or osmotic strength solutions (see for example reference 10) This paper presents the results of experiments designed to

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Special thanks are due to Drs *Preben von Magnus* and *Mogens Volkert* Various parts of this investigation were furthered through the technical assistance of Miss *A G Dyring Petersen*

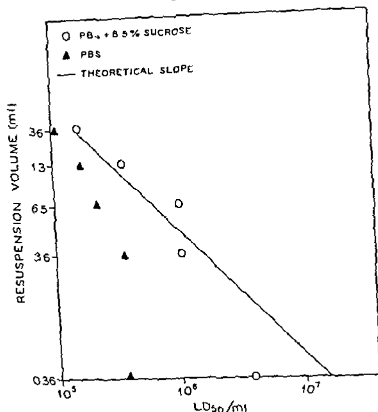


Fig 1

The effect ...

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sedimented (condition 2 Table 1) and resuspended in PBSS Here the drop was from 4 5 to less than 0 5

Fluorocarbon extractions Typical results are presented in Table 2 It is apparent that one treatment with F113 caused no loss in infectivity but another similar treatment invariably led to a large drop in titre It made no difference whether the first F113 exposure was carried out before or after sedimenting the virus However virus treated with F114 lost none of its infectivity after as many as six treatments before or after the virus had been sedimented and resuspended in PBSS In a manner similar to the results encountered when resuspending sedimented LCM virus PBSS was found to be a vital part of the

sults from a typical experiment can be seen in Fig 1 Only with PBS + 8.5 per cent sucrose (hereafter referred to as PBSS) could it be shown that infectivity of the resuspended preparations was inversely proportional to the resuspension volume Using PBS without sucrose, the LD_{50}/ml increased only slightly as the pellet was resuspended in smaller and smaller volumes Using the same type of experiment, attempts were made to establish the optimal concentration of sucrose in the resuspension solution In summary, as good results were obtained using 17 and 34 per cent sucrose as with 8.5 per cent However, the total infectivities were considerably lower when 4.25 per cent sucrose solutions were used Experiments using 8.5 per cent sucrose adjusted to pH 6, 7, and 8 gave almost identical results, with slightly higher recoveries at pH 6 and 7 than at pH 8 Similar results, with respect to pH and recovery of infectious virus, have been found using 0.5 and 1 M NaCl

TABLE 1
Centrifugation Conditions Used to Achieve Similar Minimum Apparent Sedimentation Rates

No	S min[1]	T.I.[2] super / T.I. pellet	RPM (× 1000)	Time (min.)	Spinco rotor[3] no
1	4840	> 10	10	15	40
			10	20	SW 39
			10	17	30
2	178	< 0.1*	40	27	40
			39	34	SW 39
			30	50	30
3	76	< 0.1	40	61	40
			39	79	SW 39
			30	113	30
4	268§		25	62	SW25.1

1 S'min values (13) have been calculated for time of centrifugation at full operating speed The actual time of centrifugation as shown in the table has been determined according to the method described by *Martin & Ames* (6)
2 *Equals the term total infectivity*
3 Tubes for the rotors were filled as follows 40–11 ml SW39 5ml 30–36 ml
* Only for non-fluorocarbon treated virus
§ Based on data taken from *Pfau* (9) i e the minimum apparent sedimentation rate for viral material collected at tissue culture RbCl interfaces

Ether sensitivity tests Because the success of fluorocarbon treatment depends (in many instances) on the lipid content of a virus, the ether sensitivity of LCM was reestablished (1, 12) In the first of two experiments using tissue culture fluid, the LD_{50}'s with and without ether were 0.75 and 5.5 respectively In the second experiment, the pH of the tissue culture fluid was raised with Tris buffer from 6.7 (as in the first experiment) to 7.5 Here the titres were 3.25 with, and 5.00 without, ether Another experiment was done with virus which was

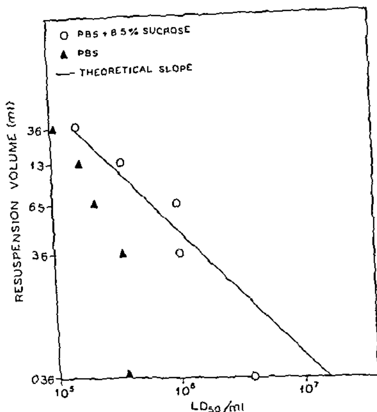


Fig 1

The effect of the resuspending solution on recovery of infectivity from sedimented LCM virus These are the results of a typical experiment carried out as follows ... of ten ... cultur ... condit ...

sedimented (condition 2, Table 1) and resuspended in PBSS Here the drop was from 4 5 to less than 0 5

Fluorocarbon extractions Typical results are presented in Table 2 It is apparent that one *treatment* with F113 caused no loss in infectivity, but another similar *treatment* invariably led to a large drop in titre It made no difference whether the first F113 exposure was carried out before *or after* sedimenting the virus However, virus treated with F114 lost none of its infectivity after as many as six treatments before or after the virus had been sedimented and resuspended in PBSS ...

ing ...

fluorocarbon procedure Although tissue culture fluid could be treated with F114 a number of times with no decrease in infectivity, once the virus was sedimented *the omission of sucrose in the resuspending solution* led to a marked drop in infectivity after the second F114 extraction

Partial purification scheme In Table 3 the results of a typical experiment, combining differential centrifugation and fluorocarbon extraction, are presented Initially the 230–300 mμ absorption spectrum of the low speed supernatant was that of a typical protein solution

TABLE 2

Effect of Fluorocarbons on LCM Virus Infectivity

Treatment before sedimentation	% recovery after				
	Sedimentation	*1st F113* treatment	*2nd F113* treatment	*1st F114* treatment	*2nd F114* treatment
—	>90	>90	<5	>90	>90
F113	>90	<5	<5	>90	<10
F114	>90	>90	<5	>90	>90

This table is a summary of the results of over 20 experiments using *F113* and *F114* The general protocol was to subject tissue culture fluid to low speed centrifugation (condition 1 Table 1) followed by homogenization with 0 25 volumes of Freon After sedimentation virus was resuspended in PBSS and treated twice with either F113 or F114 as described in the Material And Methods section

TABLE 3

Partial Purification of LCM Virus by Differential Centrifugation and Fluorocarbon Techniques

Sedimentation condition[1]	ID_{50}/ml × 10^6	Vol ml	Total infectivity × 10^6	280 mμ / 260 mμ	Total 280 mμ OD (× 10)	Total inf / OD 280 mμ / 10^6	Purification factor[3]
1S	3 33	432	1420	1 51	750	0 19	
1P	1 04	120	125				–
2S	0 0186	432	8 05			–	
2P	58 7	25	1470	1 27	18 8	7 81	41
2P*	58 7	25	1470	1 21	6 1	24 2	127
2P*	58 7	25	1470	1 07	4 32	34	178
3S	3 33	11	82 5§	–			
3P	104	2 5	1040§	0 875	2 18	48	252

1 S and P refer to the supernatant and pellet resulting from centrifugation under conditions 1 2 and 3 as described in Table 1

2 Optical density as measured in the Zeiss PMQ II spectrometer

3 The result of the division of the original value (1S) of Total infectivity/OD 280 mμ into the same type of value found at various stages of purification

* The two *fluorocarbon* steps at outlined in the Material And Methods section

§ Calculated for the entire 25 ml of suspension not just the 11 ml which were centrifuged

12 tubes for the Spinco rotor No 30 were filled with *LCM* tissue culture *fluid* and spun at low speed The supernatants were decanted and recentrifuged at high speed After treating the resuspended pellets with fluorocarbon the aqueous phase was centrifuged at high speed

After sedimentation of the virus a purification factor of over 40 was obtained and this was raised 4 to 5 fold by 2 fluorocarbon treatments At this point under the previously established conditions high speed centrifugation led to only 50 per cent of the virus being pelleted To achieve 90 per cent efficiency again it was necessary to increase centri fugation conditions so that particles with S rates of 76 or over were pelleted At this point the virus was purified by a factor of over 250 and the UV absorption spectrum of the solution now resembled that of a typical nucleoprotein

DISCUSSION

It has been possible using *Trautman* s system (13) to associate in fectious LCM virus with particles having definite physical properties In this way once S rates were established for the low and high speed differential centrifugation cycles (Table 1) it was possible to achieve equivalent conditions using any of the Spinco rotors (this cannot be done simply by using similar G forces) Due to the previously ment ioned assumptions necessary to calculate S rates from the results of *Smadel et al* (11) a rigorous comparison with our finding is not pos sible Our S rate for the low speed cycle was 4840 vs 15 000 as cal culated from the literature The value for our high speed cycle was 178 vs 500 (or 77o depending on which of the two conditions of centri fugation is used) In only one experiment have we subjected a 10 per cent sp een suspension to low speed centrifugation (condition 1 Table 1) and then 50 per cent of the virus was pelleted It seems logical to assume that virus in organ extracts is surrounded by more faster sedimenting material than virus grown in tissue culture The change in the sedimentation rate[1] of virus after fluorocarbon treatment (Table 3) again shows that as the virus becomes more purified its S rate de creases

One of the most obvious explanations for the data presented in Fig 1 is to assume that sedimented virus forms aggregates It appears that this phenomenon (in theory) can be completely reversed if the pellets are resuspended (using low ionic strength buffers) to the volume of their supernatants As the resuspension volume becomes smaller the reversal also decreases Quite different results were obtained when high ionic or osmotic strength solutions were used Then aggregation seemed to be completely *reversed* no matter what the volume of the re

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	Sedimentation	1st F113 treatment	2nd F113 treatment	1st F114 treatment	2nd F114 treatment
—	> 90	> 90	< 5	> 90	> 90
F113	> 90	< 5	< 5	> 90	< [illegible]0
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This table is a summary of the results of over 20 experiments using F113 and F114 The general protocol was to subject tissue culture fluid to low speed centrifugation (condition 1, Table 1) followed by homogenization with 0 25 volumes of Freon After sedimentation virus was resuspended in PBSS and treated twice with either F113 or F114 as described in the Material And Methods section

TABLE 3

Partial Purification of LCM Virus by Differential Centrifugation and Fluorocarbon Techniques

Sedimentation condition[1]	ID₅₀ ml × 10⁶	Vol ml	Total infectivity 10⁶	280 mμ / 260 mμ	Total 280 mμ OD[2] (× 10)	Total inf OD 280 mμ 10⁶	Purification factor[3]
1S	3 33	432	1420	1 51	750	0 19	
1P	1 04	120	125				–
2S	0 0186	432	8 05	–			
2P	58 7	25	1470	1 27	18 8	7 81	41
2P*	58 7	25	1470	1 21	6 1	24 2	127
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1 S and P refer to the supernatant and pellet resulting from centrifugation under [illegible] in Table 1

2 [illegible] Zeiss PMQ II spectrometer

3 [illegible] original value (1S) of Total infectivity OD 280 mμ into the same type of value found at various stages of purification

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is [illegible]

th [illegible] in theory) can be completely reversed if the pellets are resuspended (using low ionic strength buffers) to the volume of their supernatants As the resuspension volume becomes smaller, the reversal also decreases Quite different results were obtained when high ionic or osmotic strength solutions were used Then, aggregation seemed to be completely reversed no matter what the volume of the re

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[1] It was thought that this increase in the time necessary to sediment the virus might be largely due to the increase in th [illegible] su[illegible] ver that of the tissue culture fluid meter and standard pycnometers relative [illegible] difference was not strikingly large—1 19 for sucr[illegible] s[illegible]lution [illegible] sedimen[illegible] in sedim[illegible] properties [illegible] dence

suspension fluid was. Invoking the aggregation theory would help to explain similar data encountered in the methanol and protamine sulphate experiments (9). It could also indicate the reason for failure to recover full activity from virus solutions concentrated by carbowax (9). That is, as the solutions became more concentrated, conditions which favored aggregation may have been created.

Although herpes simplex, an ether sensitive virus, can be purified using F113 (7), it has been reported that other ether sensitive organisms are largely inactivated by this fluorocarbon (4). This study has confirmed the ether sensitivity of LCM, and has shown that the virus will not withstand treatment with the strong lipid solvent trichlorotrifluoroethane (4). In the presence of PBSS, however, the virus can be repeatedly extracted with the relatively weak lipid solvent dichlorotetrafluoroethane. Both the infectivity and the CF antigen of LCM virus are protected by 8.5 per cent sucrose solutions (PBSS). Use of sucrose was prompted by initiation of work in this laboratory (14) dealing with preparation of high titre CF antigen. A critical stage in this recently published method (5) was homogenization of tissues in 8.5 per cent sucrose solutions. Successful preparation of many arthropod-born virus CF antigens is also dependent on the use of these solutions (3).

Only 2 F114 extractions were used in the purification scheme outlined in Table 3, because further exposure failed to decrease the protein content of the fluids (as judged by the optical density readings). We have not attempted to determine the absolute protein content and only use the 280 and 260 mμ readings in a relative way. Even though the concentration of aromatic amino acids (responsible for the absorption at these two wavelengths) differs from one protein to another, it is clear that the total amount of protein is being significantly reduced. This, combined with the shift in the absorption spectrum and the decrease in case of virus sedimentation, point to the conclusion that this type of procedure may lead to the eventual purification of the virus.

SUMMARY

Alternate low and high speed centrifugation can be used to concentrate and partially purify LCM virus. Full expression of the pelleted virus can only be achieved if resuspended in phosphate buffered saline solutions containing 8.5 per cent sucrose. Evidence is presented which suggests that this high osmotic strength solution breaks up aggregates of virus formed upon sedimentation. Furthermore, only virus suspended in this way can withstand homogenization with dichlorotetrafluoroethane (but not the more commonly used trichlorotrifluoroethane). The combination of these two techniques has led to a 250-fold purification of the virus.

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Although herpes simplex, an ether sensitive virus, can be purified using F113 (7), it has been reported that other ether sensitive organisms are largely inactivated by this fluorocarbon (4) This study has confirmed the ether sensitivity of LCM, and has shown that the virus will not withstand treatment with the strong lipid solvent trichlorotrifluoroethane (4) In the presence of PBSS, however, the virus can be repeatedly extracted with the relatively weak lipid solvent dichlorotetrafluoroethane Both the infectivity and the CF antigen of LCM virus are protected by 8 5 per cent sucrose solutions (PBSS) Use of sucrose was prompted by initiation of work in this laboratory (14) dealing with preparation of high titre CF antigen A critical stage in this recently published method (5) was *homogenization of tissues in 8 5 per* cent sucrose solutions Successful preparation of many arthropod born virus CF antigens is also dependent on the use of these solutions (3)

Only 2 F114 extractions were used in the purification scheme outlined in Table 3, because further exposure failed to decrease the protein content of the fluids (as judged by the optical density readings) We have not attempted to determine the absolute protein content and only use the 280 and 260 mμ readings in a relative way Even though the concentration of aromatic amino acids (responsible for the absorption at these two wavelengths) differs from one protein to another, it is clear that the total amount of protein is being significantly reduced This, combined with the shift in the absorption spectrum and the decrease in ease of virus sedimentation, point to the conclusion that this type of procedure *may lead to the eventual purification of the virus*

SUMMARY

Alternate low and high speed centrifugation can be used to concentrate and partially purify LCM virus Full expression of the pelleted virus can only be achieved if resuspended in phosphate buffered saline solutions containing 8 5 per cent sucrose Evidence is presented which suggests that this high osmotic strength solution breaks up aggregates of virus formed upon sedimentation Furthermore, only virus suspended in this way can withstand homogenization with dichlorotetrafluoroethane (but not the more commonly used trichlorotrifluoroethane) The combination of these two techniques has led to a 250-fold purification of the virus

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otherwise stated—20 per cent activator serum which had been heat treated at 56° C for 30 minutes

The cultures Standardized cultures of HeLa cells in Gey chambers were used (5) During the outgrowth phase the cells were cultured in Hanks solution containing lactalbumin hydrolysate, antibiotics and activator serum in the concentrations mentioned above The outgrown culture consisted of a circular monolayer containing 2.9×10^5 to 5.5×10^5 cells

The immune sera Human sera from patients with suspected toxoplasmosis, which had been sent to the laboratory for diagnostic purposes were employed Only sera with a dye test titre of 4000 or more and a titre in the complement fixation tests of 64 or more were used The sera were stored at —70° C.

The activator Two batches of fresh human serum, stored at —70° C were used throughout the experiments These two sera obtained from healthy donors, were negative used undiluted in the dye tests and the complement fixation tests performed They enabled a positive reference serum to react with a constant dye test titre in the control tests included

The cell culture test The parasite suspension was mixed with an equally large volume of serum or preparation of serum to be tested The mixture was incubated at 37° C for one hour A set of three cultures was inoculated with the mixture each culture receiving 0.4 ml as replacement for the culture medium The cultures were incubated at 37° C for 19 hours and read (6)

The reading of the cell culture test At the readings parasites and cells in 10 different microscope fields of each culture were studied using phase contrast microscopy and a 40 × objective

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Th [illegible] to the concentration of *infective parasites has been* [illegible]

The dye test [illegible] titres obtained [illegible] conversion of t[illegible] When the DT was used for studies of the effect on the parasites the percentage [illegible] para[illegible]

T[illegible] the [illegible] g[illegible] ser[illegible] haemolysis

Assay of haemolytic complement The number of C'H50 per ml of serum was assayed according to *Kabat & Mayer* (10)

Treatment with [illegible] trea[illegible]

[illegible] *and R2 serum* Separation of C'1 *or* C'2 from the activator serum was performed on Sephadex® G 25 dextran gel column according to *Fjellström* (12) Fraction 1 obtained in the *procedure* for obtaining R1 was used as the R1 serum and the second portion of the precipitate *according to* the procedure for preparation of R2 was dissolved in veronal buffer and used as the R2 serum They were tested for absence of C'1 and C'2 respectively

EXPERIMENTS

Infectivity and Stainability of Toxoplasma after Exposure to Immune Serum and Activator

Samples of a human immune serum, of activator serum and of the 1:1 mixture of the immune serum and the activator (ISAM) were used unheated or heated at 56° C for 30 minutes Three ml aliquots of the

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THE EFFECT OF IMMUNE SERUM AND ACTIVATOR ON THE INFECTIVITY OF TOXOPLASMA GONDII FOR CELL CULTURE

By

ERIK LYCKE, EBBA LUND, ÖRJAN STRANNEGÅRD and ENEVOLD FALSEN

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In the toxoplasma dye test (1) the effect of antiserum on the toxoplasma parasite is elicited only in the presence of a so-called activator or accessory factor from normal human serum. The activation of the antiserum in the dye test by the activator may be abolished by heat treatment (1).

The presence of thermolabile serum components is considered necessary also for obtaining a neutralisation of the infectivity of the toxoplasma parasite by specific antibodies. In fact the antibodies demonstrable by the dye test are generally considered the same as the neutralizing antibodies. This opinion is based on evidence for specificity of the dye test reactive antibodies, the correlation between degree of immunity and serum dye test titres, and on observations that heated toxoplasma immune sera require activator to prevent infection in experimentally infected animals (2, 3).

In 1949 *MacDonald* (4) claimed, however, that a neutralizing effect of heated antiserum was demonstrable also in the absence of activator. When a new cell culture method (5, 6) was available for titration of the infectivity of toxoplasma it was considered of interest to use this method to examine the reactions of parasites to various preparations of immune serum and activator.

MATERIAL AND METHODS

The parasites. The preparation of suspensions containing parasites of the RH strain of *Toxoplasma gondii* has been described previously (5). Standardized suspensions containing 5.3×10^{5} to 16.4×10^{6} parasites per ml were made in Hanks balanced salt solution to which were added 0.5 per cent lactalbumin hydrolysate, 100 I.U. penicillin, 100 gamma streptomycin, $NaHCO_3$ to obtain a pH of 7.9 and—if not

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The skillful technical assistance of Miss *Mona Thorstensson* and Mrs. *Marie Louise Persson* is gratefully acknowledged.

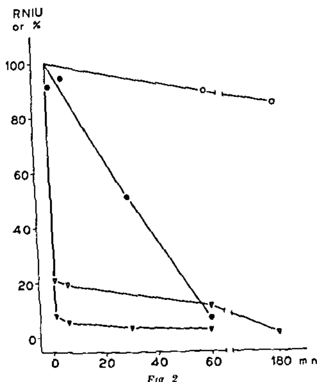


Fig 2

The effect of heat treatment on mixtures of immune serum and activator (ISAM) determined by the cell culture test and the dye test
The relative number of infective units (RNIU triangles) and the percentage of parasites with unchanged stainability (% circles) are plotted against incubation time at 37° C The filled symbols represent the results obtained with unheated ISAM open symbols with heat treated ISAM

If the ISAM was heated no effect on the stainability of the parasites was demonstrable (Fig 2)

The infectivity was found more sensitive to contact with immune serum than the stainability of the parasites was The lowest RNIU, *i e* the most effective inactivation of the parasites was obtained with unheated ISAM In the experiment illustrated by Fig 1 87 per cent of the parasites were inactivated 5 minutes after the parasites had been added to the ISAM In fact the neutralization seemed to occur at the mixing One minute of exposure to ISAM was found sufficient for neutralization of 80 per cent or more of the parasites (Fig 2)

A considerable antiparasitic activity was found also in the heat treated ISAM and in heated immune serum Fig 2 shows that almost as good an effect was achieved with heated ISAM as with the unheated serum mixture Thus in one minute 79 per cent of the parasites were inactivated with the heated ISAM whereas the corresponding percentage with unheated ISAM was 92

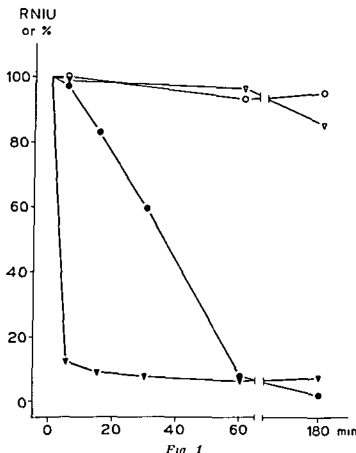


Fig 1

The effect of heat-treated activator or mixtures of immune serum and activator (ISAM) on toxoplasma as determined by the cell culture test and the dye test The relative number of infective units (RNIU triangles) and the percentage of parasites with unchanged stainability (% circles) are plotted against incubation time at 37° C The filled symbols represent the results obtained with ISAM, open symbols with heat treated activator

parasite suspension were mixed and incubated with equally large volumes of the serum samples After various incubation times at 37° C, samples of the mixtures were drawn and inoculated, each into a set of cultures These were read after incubation for 19 hours and the RNIU was registered In addition the samples were tested in the DT by counting the number of stained parasites out of 100 to 150 parasites per sample The results obtained are presented in Figs 1 and 2 by plotting the RNIU and the percentage of stained parasites against the incubation time in minutes

The heated activator serum alone had no demonstrable inactivating effect on the parasites Thus, whether tested by the cell culture method or in the DT, about 90 per cent of the parasites were still unaffected after incubation at 37° C for three hours (Fig 1)

In the presence of unheated ISAM the number of stained parasites decreased proportionally with the time of exposure After one hour at 37° C only a few per cent of the parasites showed unaffected stainability

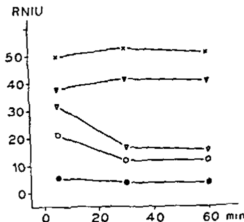


Fig 3

The effect of treatment with heat NH₄OH and zymosan on mixtures of immune serum and activator (ISAM) determined with the cell culture test
The relative number of infective units (RNIU) is plotted against incubation time at 37° C.

▼

● untreated

The largest reduction of activity seemed to be achieved when both absorption with zymosan and subsequent heat-treatment of the serum samples were used The treatment of ISAM with NH_4OH alone caused a reduction which was equally large as that found after exposure of ISAM to heat

The results summarized in Fig 3 illustrate that the different treatments of ISAM also seemed to result in a slowing down of the rate of neutralization Thus, while the untreated ISAM rapidly neutralized the parasites the neutralization caused by treated ISAM proceeded more slowly

Table 2 shows that the residual antiparasitic activity of serum after one absorption with zymosan could be almost completely removed by repeating the absorptions No difference was noticeable if the absorptions were performed at 37° C or at 19° C Unlike the effect obtained by repeating the absorptions, prolongation of the exposure of ISAM to heat or NH_4OH caused no or insignificant additional reduction of the antiparasitic activity

Attempts to restore the antiparasitic activity of ISAM, reduced by the absorptions with zymosan, are accounted for in Table 3 showing the results obtained when activator, untreated or absorbed with zymosan, was used in combination with zymosan-absorbed ISAM or immune serum The DT and cell culture tests were performed simultaneously

A restitution of activity was achieved by the addition of activator to

In both the DT and cell culture tests a small percentage of the parasites remained unaffected even after prolonged treatment with antiserum

The Antiparasitic Activity of Mixture of Immune Serum and Activator Treated with Heat, Ammonium Hydroxide and Zymosan

A human immune serum was mixed 1 1 with the activator serum Samples of the ISAM were treated according to Table 1 Thus, they were submitted to heat-treatment at 56° C for 30 min, to treatment with NH_4OH and to absorption with zymosan at 37° C All samples were finally dialysed at +4° C against Hanks' solution Differences in volume noted between the samples after the dialysis were adjusted by adding Hanks' solution so that all samples became equally diluted The final dilution of the samples corresponded to a factor of 0 3

TABLE 1

Results of Cell Culture Tests of Mixtures of Immune Serum and Activator (ISAM) Treated with Heat NH_4OH and Zymosan

Sample	Treatment			RNIU
	Heat	NH_4OH	Zymosan	
A	—	—	—	9 8
B	+		—	27 2
C	+	+	-	32 0
D	+	—	+	57 7
E	—	+		34 3
F	—	+	+	53 3
G	—		+	47 8
H	+	+	+	56 3
Activator only	+	—	—	73 6

A serum-free suspension of parasites was used The samples of ISAM and a sample of heated activator serum each received a portion of the toxoplasma suspension The mixtures of equal parts of serum sample and parasite suspension were then incubated at 37° C for one hour and inoculated into cultures The RNIU for each inoculum was determined and listed in Table 1

After incubation with heated activator serum alone or with the untreated sample (A) of the ISAM the RNIU-values found were 73 6 and 9 8, respectively, indicating the difference in the relative number of parasites with unaffected capacity of penetration before and after exposure of the parasites to ISAM If these figures are further compared with the results obtained with the treated samples (B H) of ISAM it is obvious that the treatments with heat NH_4OH and zymosan reduced but did not eliminate the antiparasitic activity of the ISAM Absorption with zymosan removed the anti toxoplasma activity most effectively

The Effect of Adding Guinea Pig Complement or Human R1- or R2 Serum to Heat-Treated Immune Serum or to Heat-Treated ISAM

Five 0 35 ml portions of an immune serum were taken Two of these were mixed with equal volumes of diluted activator and two with equal volumes of a guinea pig serum diluted 1/20 in Hanks' solution The haemolytic activity of the activator and of the diluted guinea pig serum corresponded to 4 C'H50 units per ml One of each type of serum mixture was heated at 56° C for 30 min and tested for the absence of haemolytic activity To the fifth portion of immune serum heated activator was added followed by unheated, diluted guinea pig serum

TABLE 4

Effect in Cell Culture Test of Addition of Guine Pig Serum to Heat Treated Immune Serum

Sample	RNIU
Heated activator	50 5
Heated guinea pig serum	47 0
Heated immune serum + [illegible]	13 3
Heated immune serum + [illegible]	16 6
Heated immune serum + [illegible]	28 5
Heated immune serum + [illegible]	33 0
Heated immune serum + [illegible]	13 3

TABLE 5

Effect in Cell Culture Test of Addition of R1 and/or R2-Serum to Heated Immune Serum

Sample	RNIU
Heated activator	47.2
Heated immune serum + unheated activator	11 3
Heated immune serum + heated activator	19 1
Heated immune serum + heated activator + R1 serum	17 7
Heated immune serum + heated activator + R2 serum	18 6
Heated immune serum + R1 serum + R2 serum	11 4

The five types of mixtures and also one sample of heated activator and a heated dilution of guinea pig serum were each mixed with equal volumes of a serum-free suspension of parasites After incubation at 37° C for one hour the mixtures were inoculated into cell cultures After incubation for 19 hours the cultures were read and the RNIU values were recorded The results are listed in Table 4 The thermolabile components of the activator serum could be fully substituted by the thermolabile factors of the guinea pig serum

In another experiment the ability of R1- and R2-sera to replace heat-treated activator was investigated As in the preceding experiment a

the zymosan-absorbed ISAM. This effect was discernable in both DT and cell culture tests. Likewise it could be observed that absorption of immune serum with zymosan caused no marked reduction in its anti-toxoplasma effect since an evident antiparasitic activity was noticeable in the tests when the absorbed immune serum was mixed with activator. Absorption of the activator with zymosan substantially removed its activating capacity on immune serum as demonstrated by the DT whereas no such effect was demonstrable with the cell culture test.

In an additional experiment parasites were incubated with zymosan-treated or untreated activator. The RNIU obtained with the zymosan-treated activator was 64.0 while with untreated activator the RNIU was 47.2.

TABLE 2

Results of Cell Culture Tests of Mixtures of Immune Serum and Activator (ISAM) Treated with Heat, NH_4OH and Zymosan for Different Periods of Time

Serum	Treatment	Time in min	RNIU
Activator only	Heated	30	50.2
ISAM	Heated	30	32.6
ISAM	Heated	90	32.7
ISAM	NH_4OH	90	30.6
ISAM	NH_4OH	270	34.7
ISAM	Zymosan 37° C	1 × 60	37.5
ISAM	Zymosan 37° C	2 × 60	41.8
ISAM	Zymosan 37° C	3 × 60	47.1
ISAM	Zymosan 19° C	1 × 60	35.7
ISAM	Zymosan 19° C	2 × 60	42.8
ISAM	Zymosan 19° C	3 × 60	47.1

TABLE 3

Results of Cell Culture and Dye Tests after Absorption with Zymosan and Subsequent Addition of Activator, Immune Serum or Mixture of Immune Serum and Activator (ISAM)

Sample	Treatment	Additive	RNIU	% parasites showing unchanged stainability
Activator	Heat		55.1	98.7
ISAM	None		17.6	5.6
ISAM	Zymosan*		47.2	87.6
ISAM	Zymosan	Activator	23.3	20.5
ISAM	Zymosan	ISAM	23.8	10.2
Activator	Zymosan	Immune serum	25.7	78.2
Immune serum	Zymosan	Activator	26.5	15.4
Immune serum	Zymosan	ISAM	27.3	10.6

* The serum sample was absorbed 3 times with zymosan at 37° C.

The Effect of Adding Guinea Pig Complement or Human R1- or R2 Serum to Heat-Treated Immune Serum or to Heat-Treated ISAM.

Five 0 35 ml portions of an immune serum were taken Two of these were mixed with equal volumes of diluted activator and two with equal volumes of a guinea pig serum diluted 1/20 in Hanks' solution The haemolytic activity of the activator and of the diluted guinea pig serum corresponded to 4 CH50 units per ml One of each type of serum mixture was heated at 56° C for 30 min and tested for the absence of haemolytic activity To the fifth portion of immune serum heated activator was added followed by unheated, diluted guinea pig serum

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heat-treated immune serum was divided into five portions to which was added an equally large portion of heated or unheated R1- and R2-serum or a mixture of R1- and R2-serum The serum samples, 0 8 ml each, and in addition a sample of 0 8 ml heated activator alone were each mixed with 0 8 ml of a serum-free suspension of parasites Results obtained after titration in cell cultures are listed in Table 5

Neither the R1- or R2 serum alone could be used as a substitute for the heat-treated activator When used together, however, they were able to restore the activity and to support a maximal antiparasitic action of the immune serum

The Activity of Serum Treated with EDTA

To two 1 4 ml portions of ISAM and of activator a 0 01 M solution of sodium ethylenediamine tetra-acetate (EDTA) was added, 0 14 ml to each portion of serum Subsequently one portion of ISAM and one of activator received Mg^{++} at the same concentration as that of EDTA These four samples as well as samples of heat-treated activator and of untreated ISAM were incubated at 37° C for one hour with toxoplasma parasites and inoculated into cell cultures The RNIU was determined and the results are listed in Table 6

TABLE 6

Effect in Cell Culture Test on Serum Samples Treated with EDTA and Mg^{++}

Sample	RNIU
Heated activator	57 6
Activator + EDTA	76 6
Activator + EDTA + Mg	55 1
ISAM	25 4
ISAM + EDTA	53 0
ISAM + EDTA + Mg^{++}	37 9

Treatment of ISAM with EDTA inhibited the neutralizing activity of serum When subsequently Mg^{++} was added activity of the immune serum was again demonstrable It was, however, not only in the presence of DT reactive toxoplasma antibodies that the EDTA treatment of serum was effective More parasites were found infective for the cells after incubation with activator treated with EDTA than after exposure to the untreated activator

Other experiments showed than an effect of EDTA was demonstrable only in connection with serum Thus no difference was noted if the parasites had been incubated with untreated or with an EDTA treated serum free medium, such as Hanks solution

Absorption of Serum with Toxoplasma Parasites

In another series of experiments activator, immune serum, ISAM or zymosan-treated ISAM were *absorbed* with parasites. The absorption was performed by adding the serum sample to parasites which had been sedimented by centrifugation at 440 × g for 20 minutes. The parasites were resuspended in the serum and incubated at 37° C for one hour. The tubes containing the specimens were shaken gently during the incubation to keep the parasites in suspension. After the incubation the parasites were packed together by centrifugation at 50,000 × g for 15 minutes and the serum was pipetted off. The effect of absorption was studied by mixing the absorbed serum sample with parasites which thereafter were observed in DT and cell culture tests.

A sample of the suspension of parasites in serum was, in addition, drawn before the parasites and serum were separated by high speed centrifugation. The parasites in this sample were removed from the serum and washed two times in a serum free medium by centrifugation at 440 × g each time for 20 minutes. The parasites were then exposed to activator or immune serum and studied in the DT to reveal whether toxoplasma antibodies were attached to the parasites.

TABLE 7

Results in Dye Test and Cell Culture Test of Absorption of Activator with Toxoplasma Parasites

Sample	Activator absorption	% parasites with unchanged stainability	BSR
Activator	None	88.3	51.4
Activator	Once	85.3	49.7
Activator	Twice	81.6	46.5
Immune serum* + absorbed activator	Once	14.4	23.3
Immune serum* + absorbed activator	Twice	11.7	23.3
ISAM	None	19.0	20.8

* Diluted immune serum (1:1000 in Hanks' solution) was mixed with the absorbed activator.

Table 7 shows results obtained when activator serum was used unabsorbed or absorbed once or twice with 8.8 millions of parasites per ml of serum and then tested for remaining activator effect. This was done by adding heat treated and diluted immune serum and testing the mixture on parasites. The absorptions did not remove the activating property of the activator. Moreover the parasites employed for the absorptions and then washed and exposed to heat-treated immune serum showed no change in stainability.

On the hand parasites employed for absorption of unheated immune serum and then washed in serum free medium showed conversion of

heat-treated immune serum was divided into five portions to which was added an equally large portion of heated or unheated R1- and R2-serum or a mixture of R1- and R2-serum. The serum samples, 0.8 ml each, and in addition a sample of 0.8 ml heated activator alone were each mixed with 0.8 ml of a serum-free suspension of parasites. Results obtained after titration in cell cultures are listed in Table 5.

Neither the R1- or R2 serum alone could be used as a substitute for the heat-treated activator. When used together, however, they were able to restore the activity and to support a maximal antiparasitic action of the immune serum.

The Activity of Serum Treated with EDTA

To two 1.4 ml portions of ISAM and of activator a 0.01 M solution of sodium ethylenediamine tetra-acetate (EDTA) was added, 0.14 ml to each portion of serum. Subsequently one portion of ISAM and one of activator received Mg^{++} at the same concentration as that of EDTA. These four samples as well as samples of heat-treated activator and of untreated ISAM were incubated at 37° C for one hour with toxoplasma parasites and inoculated into cell cultures. The RNIU was determined and the results are listed in Table 6.

TABLE 6

Effect in Cell Culture Test on Serum Samples Treated with EDTA and Mg^{++}

Sample	RNIU
Heated activator	57.6
Activator + EDTA	76.6
Activator + EDTA + Mg	55.1
ISAM	25.4
ISAM + EDTA	53.0
ISAM + EDTA + Mg	37.9

Treatment of ISAM with EDTA inhibited the neutralizing activity of serum. When subsequently Mg^{++} was added activity of the immune serum was again demonstrable. It was, however, not only in the presence of DT reactive toxoplasma antibodies that the EDTA treatment of serum was effective. More parasites were found infective for the cells after incubation with activator treated with EDTA than after exposure to the untreated activator.

Other experiments showed than an effect of EDTA was demonstrable only in connection with serum. Thus no difference was noted if the parasites had been incubated with untreated or with an EDTA-treated serum-free medium, such as Hanks' solution.

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In another series of experiments activator immune serum ISAM or zymosan treated ISAM were absorbed with parasites. The absorption was performed by adding the serum sample to parasites which had been sedimented by centrifugation at 440 × g for 20 minutes. The parasites were resuspended in the serum and incubated at 37° C for one hour. The tubes containing the specimens were shaken gently during the incubation to keep the parasites in suspension. After the incubation the parasites were packed together by centrifugation at 50 000 × g for 15 minutes and the serum was pipetted off. The effect of absorption was studied by mixing the absorbed serum sample with parasites which thereafter were observed in DT and cell culture tests.

A sample of the suspension of parasites in serum was in addition drawn before the parasites and serum were separated by high speed centrifugation. The parasites in this sample were removed from the serum and washed two times in a serum free medium by centrifugation at 440 × g each time for 20 minutes. The parasites were then exposed to activator or immune serum and studied in the DT to reveal whether toxoplasma antibodies were attached to the parasites.

TABLE 7

Results in Dye Test and Cell Culture Test of Absorption of Activator with Toxoplasma Parasites

Sample	Activator absorption	% parasites with unchanged staining	RNR
Activator	None	88 3	51 4
Activator	Once	85 3	49 7
Activator	Twice	81 6	46 5
Immune serum + absorbed activator	Once	14 4	23 3
Immune serum + absorbed activator	Twice	11 7	23.3
ISAM	None	19 0	20 8

Diluted immune serum (1 1000 in Hanks solution) was mixed with the absorbed activator

Table 7 shows results obtained when activator serum was used unabsorbed or absorbed once or twice with 8 8 millions of parasites per ml of serum and then tested for remaining activator effect. This was done by adding heat treated and diluted immune serum and testing the mixture on parasites. The absorptions did not remove the activating property of the activator. Moreover the parasites employed for the absorptions and then washed and exposed to heat treated ...
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heat-treated immune serum was divided into five portions to which was added an equally large portion of heated or unheated R1- and R2-serum or a mixture of R1- and R2-serum The serum samples, 0 8 ml each, and in addition a sample of 0 8 ml heated activator alone were each mixed with 0 8 ml of a serum-free suspension of parasites Results obtained after titration in cell cultures are listed in Table 5

Neither the R1- or R2 serum alone could be used as a substitute for the heat-treated activator When used together, however, they were able to restore the activity and to support a maximal antiparasitic action of the immune serum

The Activity of Serum Treated with EDTA

To two 1 4 ml portions of ISAM and of activator a 0 01 M solution of sodium ethylenediamine tetra-acetate (EDTA) was added, 0 14 ml to each portion of serum Subsequently one portion of ISAM and one of activator received Mg^{++} at the same concentration as that of EDTA These four samples as well as samples of heat-treated activator and of untreated ISAM were incubated at 37° C for one hour with toxoplasma parasites and inoculated into cell cultures The RNIU was determined and the results are listed in Table 6

TABLE 6

Effect in Cell Culture Test on Serum Samples Treated with EDTA and Mg

Sample	RNIU
Heated activator	57 6
Activator + EDTA	76 6
Activator + EDTA + Mg	55 1
ISAM	25 4
ISAM + EDTA	53 0
ISAM + EDTA + Mg^{++}	37 9

Treatment of ISAM with EDTA inhibited the neutralizing activity of serum When subsequently Mg^{++} was added activity of the immune serum was again demonstrable It was, however, not only in the presence of DT reactive toxoplasma antibodies that the EDTA treatment of serum was effective More parasites were found infective for the cells after incubation with activator treated with EDTA than after exposure to the untreated activator

Other experiments showed than an effect of EDTA was demonstrable only in connection with serum Thus no difference was noted if the parasites had been incubated with untreated or with an EDTA-treated serum-free medium, such as Hanks' solution

Absorption of Serum with Toxoplasma Parasites

In another series of experiments activator, immune serum, ISAM or zymosan treated ISAM were absorbed with parasites. The absorption was performed by adding the serum sample to parasites which had been sedimented by centrifugation at 440 × g for 20 minutes. The parasites were resuspended in the serum and incubated at 37° C for one hour. The tubes containing the specimens were shaken gently during the incubation to keep the parasites in suspension. After the incubation the parasites were packed together by centrifugation at 30 000 × g for 15 minutes and the serum was pipetted off. The effect of absorption was studied by mixing the absorbed serum sample with parasites which thereafter were observed in DT and cell culture tests.

A sample of the suspension of parasites in serum was, in addition, drawn before the parasites and serum were separated by high speed centrifugation. The parasites in this sample were removed from the serum and washed two times in a serum free medium by centrifugation at 440 × g each time for 20 minutes. The parasites were then exposed to activator or immune serum and studied in the DT to reveal whether toxoplasma antibodies were attached to the parasites.

TABLE 7

Results in Dye Test and Cell Culture Test of Absorption of Activator with Toxoplasma Parasites

Sample	Activator absorption	% parasites with unchanged stainability	BNR
Activator	None	88.3	51.4
Activator	Once	85.3	49.7
Activator	Twice	81.6	46.5
Immune serum + absorbed activator	Once	14.4	23.3
Immune serum + absorbed activator	Twice	11.7	23.3
ISAM	None	19.0	20.8

Diluted immune serum (1 : 1000 in Hanks' solution) was mixed with the absorbed activator.

Table 7 shows results obtained when activator serum was used unabsorbed or absorbed once or twice with 8.8 millions of parasites per ml of serum and then tested for remaining activator effect. This was done by adding heat treated and diluted immune serum and testing the mixture on parasites. The absorptions did not remove the activating property of the activator. Moreover the parasites employed for the absorptions and then washed and exposed to heat treated immune serum showed no change in stainability.

On the hand parasites employed for absorption of unheated immune serum and then washed in serum free medium showed conversion of

heat-treated immune serum was divided into five portions to which was added an equally large portion of heated or unheated R1- and R2-serum or a mixture of R1- and R2-serum The serum samples, 0 8 ml each, and in addition a sample of 0 8 ml heated activator alone were each mixed with 0 8 ml of a serum-free suspension of parasites Results obtained after titration in cell cultures are listed in Table 5

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A sample of the suspension of parasites in serum was, in addition, drawn before the parasites and serum were separated by high speed centrifugation. The parasites in this sample were removed from the serum and washed two times in a serum free medium by centrifugation at 440 × g, each time for 20 minutes. The parasites were then exposed to activator or immune serum and studied in the DT to reveal whether toxoplasma antibodies were attached to the parasites.

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* Diluted immune serum (1:1000 in Hanks solution) was mixed with the absorbed activator.

Table 7 shows results obtained when activator serum was used unabsorbed or absorbed once or twice with 8.8 millions of parasites *per ml* of serum and then tested for remaining activator effect. This was done by adding heat-treated and diluted immune serum and testing the mixture on parasites. The absorptions did not remove the activating property of the activator. Moreover the parasites employed for the absorptions and then washed and exposed to heat-treated immune serum showed no change in stainability.

On the hand parasites employed for absorption of unheated immune serum and then washed in serum free medium showed conversion of

stainability in 81 per cent when activator was added The corresponding per cent figures, found for parasites used for absorption of heated immune serum or zymosan-treated ISAM, were after the addition of activator 31 and 21, respectively Thus antibodies seemed to be attached poorly if the immune serum had been previously heat-treated and even less if it had been absorbed with zymosan

TABLE 8
Results in Dye Test and Cell Culture Test of Absorption of Immune Serum with Toxoplasma Parasites

Sample	Final dilution of immune serum	Immune serum absorption	% parasites with unchanged stainability	RNIU
Heated activator			94 9	59 4
Heated immune serum	1 2000	None	91 8	45 6
Heated immune serum	1 400	Once	89 3	48 0
Heated immune serum	1 800	Twice	97 5	55 1
Heated immune serum	1 1600	Thrice	92 6	53 9
Activator + absorbed immune serum	1 400	Once	11 8	26 1
Activator + absorbed immune serum	1 800	Twice	9 7	30 4
Activator + absorbed immune serum	1 1600	Thrice	13 3	38 8

It seemed thus to be difficult to remove antibodies from heat-treated immune serum by absorption with toxoplasma organisms One of the experiments performed is illustrated in Table 8 As can be seen, the DT results obtained after absorption of the immune serum and subsequent addition of activator indicated no loss of antibodies In the cell culture tests an effect on the RNIU was however demonstrable when absorption of the immune serum was repeated, each time with one million parasites per 2 ml of mixture This was most evident when the immune serum was mixed with activator after the absorption

In the presence of activator, toxoplasma antibodies were readily removed from immune serum by absorptions with parasites This could be demonstrated by the DT as well as by the cell culture tests

DISCUSSION

In the reported experiments the infectivity of toxoplasma protozoa was assayed by a cell culture method By this technique the number of parasites in the inoculum which penetrate the cultured host cells is determined Consequently the activity of the serum components studied was that affecting primarily the ability of the parasites to penetrate The term neutralization will be used in the following to describe this effect of serum on the parasites

Sabin & Feldman (1) showed that thermolabile components of the activator serum were necessary for the DT reaction to occur Later on

the complement factors C'_1, C'_2, C'_3 and C'_4 (13, 14), magnesium ions (15) and properdin (15, 16) were reported to be the components responsible for the activating effect on toxoplasma immune serum

The most effective neutralization was achieved in the present study when mixtures of immune serum and activator (ISAM) were employed Treatment with heat to destroy the thermolabile complement factors resulted in reduction of the neutralizing capacity of ISAM but its efficacy could be restored by subsequent addition of diluted normal guinea pig serum or a mixture of human R1- and R2-sera Treatment of ISAM with NH_4OH for destruction of C'_4 caused a reduction of the antiparasitic activity as did treatment with EDTA The reduction caused by EDTA could be reversed by addition of magnesium ions The largest decrease in the neutralizing capacity of ISAM was however encountered when ISAM was absorbed with zymosan, an effect which disappeared if fresh activator was subsequently supplied Thus, at least a substantial part of the effect on the infectivity of toxoplasma observed in the cell culture test seemed to be produced by antibodies which, as in the DT, required for function the presence of the serum complement factors, magnesium ions and properdin

The cell culture test revealed in addition that heated immune serum or heated ISAM possessed a considerable neutralizing capacity which was not discerneable in the DT This was a regularly observed phenomenon with all the immune sera examined but was not observed with any of the tested DT negative sera The concentration of the component(s) responsible for the activity was large enough to permit a detectable effect also with highly diluted immune sera

The observation of an effect on toxoplasma by heat treated immune serum is in agreement with that of *MacDonald* (4) He reported in 1949 that he obtained neutralization of toxoplasma infectivity tested on the chorioallantoic membranes of embryonated eggs with immune sera which had been heated at 56° C for 30 minutes

The difference between the results obtained by the DT and the cell culture test with heated immune serum is explicable by the assumption that a type of antibodies in addition to those demonstrable by DT and requiring activator, is indicated by the cell culture tests Studies employing immunoprecipitation (17, 18, 19) with toxoplasma antigens and immune sera show that there exist several antigen–antibody precipi- [illegible]

[illegible] *Strannegård* (19)

[illegible] precipitins studied

[illegible] DT or CF antibodies

If there were antibodies in toxoplasma immune serum at any appreciable concentration which did not require the presence of properdin for function and which were demonstrable by the cell culture test but not with the DT, these antibodies must have been removed from serum by absorption with zymosan Such repeated absorptions almost eliminated the anti parasitic activity of ISAM It was found, however, that

when subsequent to absorption activator serum was added, the antiparasitic activity of ISAM was restored Thus there was no evidence found that antibodies were removed from immune serum by absorption with zymosan

A difference in results with the two tests used would also be obtained if the serum properdin system itself interfered with the penetration of parasites in the absence of toxoplasma antibodies —As shown by *Gronroos* (20) and *Feldman* (15) properdin does not cause any conversion of the stainability in absence of antibody— Properdin acts in conjunction with complement and magnesium ions (21) The finding that more parasites were found to have penetrated after exposure to EDTA treated normal serum (activator) than after incubation with untreated normal serum or treated serum to which magnesium ions was added might therefore indicate an effect by properdin in the absence of specific antibody This is supported by the observation that in comparison to normal serum absorbed with zymosan the untreated serum caused some inhibition of the penetration

It is possible, however, that toxoplasma antibodies were present in the "normal" sera used in a concentration undetectable in the DT If this were the case the effect of EDTA and zymosan might be attributed to the removal of magnesium and properdin necessary for the antibody action It should be mentioned that *Osawa & Muschel* (22) consider the properdin system itself inadequate to explain the bactericidal action of normal serum They found that when a human serum was absorbed with the test organism it was inactive despite the presence of properdin and complement

The penetration-promoting capacity of EDTA could possibly also be ascribed to an effect of this substance on the host cell wall The present authors (23) have shown that purified egg-white lysozyme has a penetration promoting effect which is not mediated by serum factors A similar function of EDTA, however, is not probable as the effect of EDTA could not be demonstrated in the absence of serum

Absorption of preparations of activator, immune serum and ISAM with toxoplasma organisms indicated that antibodies combined but poorly with parasites in the absence of properdin and complement This may be inferred also from the apparently slower neutralization rate observed when ISAM had been treated with heat, NH_4OH and zymosan In the presence of activator components the avidity of the toxoplasma antibodies is obviously increased There also seems however to be an absorption of antibodies in the absence of the heat-labile activator components, resulting in inhibition of the penetration of the parasites

SUMMARY

Parasites of the RH-strain of *Toxoplasma gondii* were exposed to preparations and mixtures of activator serum and immune serum and

then tested in cell culture tests and dye tests The penetration of the parasites was inhibited if the parasites had been exposed not only to immune serum with activator but also if they had been incubated with immune serum in which the heat labile components were destroyed

As in the dye test properdin complement and magnesium ions enhanced the effect of toxoplasma antibodies in the cell culture test Some results indicated in addition that the properdin system might cause inhibition of penetration in the absence of toxoplasma antibodies

The avidity of toxoplasma antibodies seems to be increased in the presence of the activator components The difference in results obtained with the two tests may be explained as due to a higher sensitivity of the cell culture test than that of the dye test for antibodies of low avidity

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COMPLEMENT-FIXING ANTIBODIES TO ADENOVIRUS IN RABBITS AND GUINEA-PIGS TREATED WITH 6 MERCAPTOPURINE OR ε-AMINOCAPROIC ACID

By

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The suppressive effect of a drug on immune response is greatly dependent not only on dosage and timing but also on the test animal used and on the quality, amount and route of administration of the antigen 6 mercaptopurine (6 MP) is known to inhibit antibody production in several species after challenge by various antigens (for references see *Hitchings & Elion* 1963) It has been reported also that 6-MP lowers the resistance of monkeys to variola and vaccinia viruses, and of rabbits to vaccinia, but not to variola, although antibody levels are depressed (*Janssen, Marshall, Gerone & Cheville* 1962, *Janssen, Marshall, Gerone, Cheville & Convey* 1962) The present study examines the antibody response to adenovirus in rabbits and guinea-pigs treated with 6-MP

ε-aminocaproic acid (EACA), an antifibrinolytic drug, has been used in the treatment of various allergic conditions (*Mikata, Hasegawa, Igarashi, Shirakura, Hoshida & Toyama* 1959, *Yokoyama & Hatano* 1959, *Oswald* 1961 *Stacher* 1962 *Arnet, Neubauer & Schuppli* 1963, *Rotstein, Gilbert & Estrin* 1963) It has prevented anaphylaxis in experimental animals (*Okamoto* 1959, *Otto-Servais & Lecomte* 1961, *Zweifach, Nagler & Troll* 1962) reduced vascular reactions to histamine (*Lefebvre, Salmon Lecomte & van Cauwenberge* 1962), and it has also been used to prolong survival of skin and tumour transplants (*Bertelli, Bonmassar, Genovese & Trabucchi* 1963, *Bertelli & Frontino* 1963 *Gillette, Findley & Conway* 1963 a, 1963 b) EACA inhibits the tuberculin reaction in the skin (*Itoga & Yogo* 1959, *Stacher, Lowney* 1964) and also inhibits to some extent other delayed-type hypersensitivity reactions (*Wutrich, Rieder & Ritzel* 1963, *Toivanen & Toivanen* 1964) The local Shwartzman phenomenon is also inhibited by this drug in ...

20 *Gronroos P* Discussion of the paper of Fichenwald F The laboratory diagnosis of toxoplasmosis Ann N Y Acad Sci *64* 213 1956
21 *Lepow I H Pillemer L Schoenberg M D Todd E W & Wedgwood R J* The properdin system and immunity X Characterization of partially purified human properdin J Immunol *83* 428 1959
22 *Osawa E & Muschel L H* Bactericidal action of normal serum and the properdin system J Immunol *?03* 84 1960
23 *Lycke E Lund Ebba & Strannegård Ö* Enhancement by lysozyme and hyaluronidase of the penetration of Toxoplasma gondii into cultured host cells Brit J Exp Path in press

daily injection. The treatment began two days before the administration of the antigen and continued to the end of the experiment.

Statistics

The reciprocals of the antibody titres were expressed as log and the exponents were used in statistical treatment of the results. Student's *t* test was employed. For all calculations the reciprocals expressed as < 4 were considered 2.

RESULTS

The antibody response to adenovirus in rabbits treated with 6-MP or EACA are shown in *Table 1*. Statistically significantly decreased antibody level was observed almost throughout the experiment in the group treated with 6-MP. In rabbits treated with EACA the titres were about the same as in the control group, with the exception of the specimens for the 4th day. On that day, elevated antibody titres could be seen only in the controls, while in other groups no response could be observed.

TABLE 1

Antibodies to Adenovirus in Rabbits Treated with 6-MP or EACA. Days Read from the Injection of the Antigen. A Booster Dose was Given after 2 Weeks

Treatment	Rabbit no.	Reciprocal of antibody titre on different days: 2	4	8	12	19	26
6-MP 10 mg/kg/day	1	<4	4	64	64	64	128
	2	4	4	64	32	128	128
	3	4	4	128	64	256	256
	4	<4	<4	8	16	32	32
	5	<4	<4	16	16	32	32
Geometric mean		3	3*	37*	32***	74*	84
EACA 500 mg/kg/day	6	<4	4	256	256	256	512
	7	<4	4	64	32	128	128
	8	<4	<4	128	64	512	256
	9	<4	4	256	256	256	256
	10	<4	<4	128	64	256	128
Geometric mean		2	3	147	97	256	223
EACA 200 mg/kg/day	11	<4	<4	128	512	1024	1024
	12	<4	4	64	64	256	128
	13	<4	<4	128	64	256	128
	14	<4	4	128	64	256	128
	15	<4	<4	512	128	256	128
Geometric mean		2	3*	147	111	338	194
Physiological saline	16	<4	16	128	256	256	512
	17	<4	16	256	256	256	256
	18	<4	8	128	256	1024	1024
	19	<4	8	128	128	256	128
	20	4	<4	128	128	128	128
Geometric mean		2	8	147	194	294	294

The asterisks refer to the level of significance in comparison with the corresponding value in the control group. $^{*}0.05 > P > 0.02$; $^{***}P < 0.001$

from cell extracts (*Johanovsky & Škavril* 1962) and inhibits the precipitation and complement-fixation reactions (*Atchley & Bhagavan* 1962, *Bertelli et al.*). The exact mechanism at work in these processes is unknown, and for this reason we have included EACA in the present study to obtain further information about its immunological properties.

MATERIAL AND METHODS

Antigens

Antigens for immunization and complement fixation were prepared as follows: HeLa cell monolayers grown in Roux bottles in Hanks' balanced salt solution with 40 per cent human serum were washed three times with the balanced salt solution. The maintenance medium, consisting of Eagle's Minimum Essential Medium with 5 per cent horse serum and 5 per cent tryptose phosphate broth, was added and adenovirus type 5 or 6 (obtained from Dr. H. G. Pereira, National Institute for Medical Research, Mill Hill, London) was inoculated. After 3 to 5 days, when the cytopathic effect was complete, the cells were disrupted by freezing and thawing 6 times and cell debris was removed by low speed centrifugation. The antigens were titrated against positive human serum pool.

Antibody Titration

The sera were stored at —20° C until examined. The rabbit and guinea pig sera were inactivated at 60° C for ½ hour. The complement fixation was made with 0.1 ml reagent volumes. Four units of antigen (adenovirus type 5) and two full units of complement were used. After overnight fixation at +4° C the sensitized cells were added and after ½ hour of secondary incubation at 37° C the test was read. A control titration of complement and a titration of a known positive human serum were included in each test. All the sera of one experiment were titrated simultaneously.

Animals and the Administration of the Drugs

Albino rabbits (weighing 2.2–2.7 kg) and guinea pigs (weighing 0.50–0.60 kg) of both sexes were used as test animals.

The rabbits were divided into four groups of five animals. They were treated daily with 6–MP 10 mg/kg/day or with EACA 500 or 200 mg/kg/day. One group served as a control treated with physiological saline. All rabbits were immunized with two subcutaneous injections of adenovirus type 6, each injection of 3.0 ml and 2 weeks apart. The complement fixation titre of the antigen was 1:128.

The guinea pigs were divided into six groups as follows:

Group A 16 animals treated with 6–MP 10 mg/kg/day
Group B 17 animals treated with EACA 200 mg/kg/day
Group C 16 animals treated with physiological saline

These three groups were immunized with one intraperitoneal and one subcutaneous injection of adenovirus type 5 (1.5 + 1.5 ml) and 2 weeks later a booster dose of 1.5 ml was given intraperitoneally. The complement fixation titre of the antigen was 1:64.

Group D 15 animals treated with 6–MP 20 mg/kg/day
Group E 15 animals treated with EACA 600 mg/kg/day
Group F 15 animals treated with physiological saline

These three groups were immunized with one intraperitoneal and one subcutaneous injection of adenovirus type 6 (3.0 + 3.0 ml). No booster dose was given. The complement fixation titre of the antigen was 1:32.

The blood specimens of the animals were taken at intervals of 4 to 7 days, of the rabbits from ear veins and of the guinea pigs by cardiac puncture. In general the same guinea pigs were not bled on consecutive occasions.

6–MP (Juva, Milan) was dissolved in 1 N NaOH at a concentration of 100 mg/ml. This solution was diluted in physiological saline to contain 5 mg/ml. The solution was prepared daily. EACA (Epsikapron®, Kabi, Stockholm) was used at a concentration of 100 mg/ml. Both drugs were administered intraperitoneally in a single

DISCUSSION

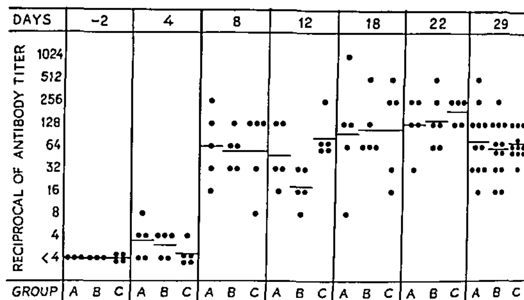
In the present study only a slight depression of the antibody production was observed in the rabbits treated with 6-MP. The dosage used was not large but a total inhibition has been produced with an even smaller dose, e.g. by *Nachtigal & Feldman* (1963) using human and bovine serum albumin as antigens. Our results show, however, that antibody response to adenovirus can be influenced by 6-MP, and experiments with varying doses of antigen should surely give different results. There is no reason to assume that viral antigens should be different from other antigens in so far as the effect of 6-MP on antibody production is concerned. *Janssen*'s failure to lower the resistance of rabbits to variola virus is probably unconnected with the effect of 6-MP on the viral antibodies, as pointed out by *Hitchings & Elion*.

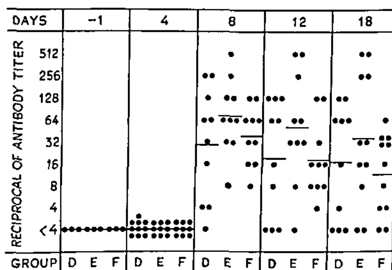
No effect of 6-MP on the adenovirus antibodies was observed in the guinea pigs. This is in accord with earlier observations that guinea pigs are relatively resistant to 6-MP (see *Hitchings & Elion*, *Maguire & Steers* 1963).

EACA obviously has very little if any effect on the antibody response to adenovirus in rabbits or guinea pigs. The only time when some effect might perhaps have been discernible was on the 4th day in the rabbit specimens (Table 1). We are not able to assess whether or not this finding has any significance, nor is it possible to say if it were caused by delayed antibody synthesis or by the effect of EACA in preventing the antibodies from being detectable. In experimental allergic encephalomyelitis *Wulrich et al.* have found a slight reduction in the production of circulating antibodies in rabbits treated with EACA. It is known that the administration of a single amino acid such as L-phenylalanine is able to block the antibody synthesis (*Ryan & Carver* 1964). Bearing these facts in mind it may be concluded that diminution of the active antibody level by EACA cannot at present be ruled out from the search for the mechanism by which this drug depresses various allergic and hypersensitivity reactions. The antiproteolytic action of EACA (*Abe & Sato* 1959, *Alkjaersig, Fletcher & Sherry* 1959, *Igawa, Watanabe, Amano & Okamoto* 1959, *Lewis* 1963), however, certainly has a role in this mechanism. Proteolytic enzymes are involved in allergic and hypersensitivity phenomena (see *Ungar & Hayashi* 1958), and on this basis, too, the immunological effects of EACA can be explained. Further, EACA reduces the complement level of serum (*Bertelli et al.*), and this can also be attributed to the depression of the immune response (*Osler* 1961).

SUMMARY

The primary antibody response to adenovirus, measured by the complement fixation reaction, is to some extent suppressed by 6-mercaptopurine in rabbits but not in guinea pigs. ε-aminocaproic acid has very





A 6-MP 10 mg/kg/day
B EACA 200 mg/kg/day
C physiological saline

D 6-MP 20 mg/kg/day
E EACA 600 mg/kg/day
F physiological saline

Fig 1

Antibodies to adenovirus in guinea-pigs treated with 6 MP or EACA Each spot represents one guinea-pig The horizontals show the geometric mean Days read from the injection of the antigen *In groups A C a booster dose was given after 2 weeks*

In the guinea-pigs, 6-MP or EACA had no apparent effect, in either the bigger or the smaller dosage (Fig 1) Decreased titres were to be observed only on the 12th day, in the group treated with 200 mg kg day of EACA, but specimens for the adjacent times did not differ from the controls

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little, if any, effect on the adenovirus antibody level in rabbits and guinea-pigs

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The Department of Bacteriology, Karolinska Institutet Stockholm Sweden

PREPARATION OF BIOLOGICALLY ACTIVE FRACTIONS OF *SALMONELLA TYPHIMURIUM*

3 *Extraction of Immunogenic Components*

By

L Edebo and T Holme

Received 28 vii 64

During studies of the influence of cultivation conditions on the biologically active components of *Salmonella typhimurium* experiments concerned with the extraction of the immunogenic antigens were carried out Cell walls were prepared by mechanical disintegration of the cells and subsequent washings Since it was found that most of the immunogenic activity was present in the washing fluids, further experiments were directed to the determination of convenient extraction methods for those antigens from disintegrated materials as well as from whole cells

Work on *Salmonella enteritidis* (*Ribi et al* 1959), *Salmonella typhi* (*Carey & Baron* 1959), and several other pathogenic gram-negative bacteria has conclusively shown that the major immunogenic antigens are located in the cell walls of these organisms There is no reason to doubt, that this is also the case for *Salmonella typhimurium*, but the fact that the immunogenic activity is so easily extracted, makes it extremely difficult to prepare cell wall fractions of reasonable purity, which retain their immunogenicity

The pronounced variability in the yield of biologically active substances from different gram negative bacteria was pointed out by *Nowotny et al* (1963) He applied six different extraction methods for endotoxins on *Serratia marcescens, Salmonella typhi* and *Escherichia coli* The toxicity of the preparations varied considerably A tri-chloro-acetic acid extract from *S marcescens* and *S typhi* was highly toxic but from *E coli* of very low toxicity, while a phenol water extract from *S typhi* and *E coli* proved highly toxic but only slightly toxic from *S marcescens* In the light of these findings it is not surprising that differences in the yield of biologically active components occur when different *Salmonella* types are extracted

Our thanks are due to Mrs *Kerstin Magnusson* and Miss *Berit Lindholm* for their skilled technical assistance This work was aided by a grant from the *Swedish State Medical Research Council*

METHODS

Strains The following strains were obtained from the State Bacteriological Laboratory Stockholm *Salmonella typhimurium* 395M *Salmonella bonariensis* (I S 145) A strain of *Escherichia coli* B kept in the department was used in one experiment A stable R mutant was isolated from a aged broth culture of the

[illegible] ...erwise stated cultures were grown in a [illegible] in aerated submerged culture as described

Disintegration procedure The press described by *Edebo & Holme* (1961) was used It was operated at —20° C and each sample was treated in three passages. Washings of the disintegrated material in different salt solutions were performed in the high speed attachment of a IEC-PR 2 centrifuge at +4° C and 25 000 × g

Dry weight determinations Cells were collected by centrifugation and washed once in 0 01 M phosphate buffer and dried overnight at 100° C. The weight values were corrected for the s lids in the washing fluid Extracts were dialyzed overnight against running cold water transferred to a tube and dried by blowing air into the tube immersed in a water bath at 80° C However, before the determination of dry weights remaining traces of moisture were removed by heating the extracts at a 100° C overnight

Animal tests White mice of 8 12 g were used Toxicity tests were carried out as previously described (*Holme & Edebo* 1961) In the protection test five groups of ten mice were injected intrapertioneally with dilutions of the test material from each sample An intraperitoneal challenge was given ten days after immunization The challenge dose was 10^{4} viable cells per mouse As more than 90 per cent of the resultant deaths occurred 5 10 days after the challenge was given the total number of deaths up to the tenth day was chosen as the basis for the calculation of PD_{50} The calculation was made according to *Reed & Muench* (1938) A control group was immunized with a heat killed standard vaccine[1] prepared from *Salmonella typhimurium* 395 M each time a test was carried out A non immunized group was also included as a challenge control The PD_{50} values of the control groups during the experimental period are given in Table 5

RESULTS

The first experiment was designed to determine the location of the toxic and immunogenic components in cells of *Salmonella typhimurium* The cells from a one litre culture, giving a yield of 10 g on a dry weight basis were inactivated by adding merthiolate to give a concentration level of 0 01 per cent, centrifuged and resuspended in 0 15 M sodium chloride This suspension was then centrifuged and the supernatant tested for the biological activities of any high molecular weight substances released The cell paste was first frozen and then the cells disintegrated by freeze pressing (*Edebo & Holme* 1961) The disintegrated material was then washed three times in 0 1 M phosphate buffer, three times in 1 M sodium chloride and three times in distilled water Electron microscopy showed that the preparation consisted of cell wall fragments, apparently free of cytoplasmic materials (cf *Edebo & Holme* 1961) A duplicate experiment was carried out using cells from a second culture As can be seen from Table 1 the cell wall fraction showed comparatively low toxicity and immunogenicity The materials released into the washing fluids had a higher immunizing capacity and it was

[1] The standard vaccine was kindly prepared by Dr *H Billaudelle*, the State Bacteriological Laboratory Stockholm Sweden

repeatedly found that the resultant solution from the first washing in 1 M sodium chloride, although containing only very small amounts of material, had the highest immunogenic capacity

TABLE 1

Toxicity and Immunogenicity of Cell Wall Preparations and Extracts of Salmonella typhimurium

		LD_{50} (mg)	PD_{50} (mg) 10^{-5}
M9 H-S1	0 15 M sodium chloride	2 5	5 3
M9-W-S1	0 1 M phosphate buffer	>4	4 5
M9 W-S4	1 M sodium chloride	3 2	0 3
M9 W		>4	20
M10-W-S1	0 1 M phosphate buffer	>4	10
M10-W-S4	1 M sodium chloride	1 2	0 6
M10-W		>4	13

Cells from two cultures M9 and M10 were used for the preparation of cell walls M9 H-S1 is the supernatant from the first washing preceding the disintegration W-S1 is the supernatant from the first washing in phosphate buffer and W-S4 the first in sodium chloride M9-W and M10-W are the cell wall fractions
All washings were carried out in the cold

TABLE 2

Toxicity and Immunogenicity of Extracts of Salmonella typhimurium

	Dry weight (mg ml)	LD_{50} (mg)	LD_{50} (mg) $\times 10^{-5}$
M21 – Dist water	0 8	0 12	0 3
0 15 M sodium chloride	1 2	0 15	0 2
1 M sodium chloride	1 5	0 15	0 1
2 M sodium chloride	0 9	0 21	0 1
0 2 M phosphate buffer pH 6	2 2	0 54	0 5
0 2 M phosphate buffer pH 7	3 0	0 71	5 0
0 2 M phosphate buffer pH 8	2 6	0 47	5 0
M31 – 1 M sodium chloride			1 2
M33 – 1 M sodium chloride			7 9
M38 – 1 M sodium chloride			2 5
M39 – 1 M sodium chloride			7 9

Whole cells from cultures of *Salmonella typhimurium* 395M were suspended in different salt solutions to a concentration of 10 mg dry weight per ml and heated to 70° C for 30 minutes

In the next experiment (Table 2) whole cells from a one litre culture were extracted The culture was centrifuged while viable and the cells resuspended at a concentration of 10 mg dry weight per ml in different salt solutions and then heated to 70° for 30 minutes In both distilled water and sodium chloride solutions about 10 per cent of the cell weight was extracted The extracts showed a good immunizing effect Extracts in phosphate buffers at pH 7 and 8 gave lower proctection, but at pH 6 the difference from the sodium chloride extracts was not significant When extraction with 1 M sodium chloride was performed on materials

TABLE 3

Immunogenicity of Cells and Extracts of Salmonella typhimurium 395 M

	LD50 (mg) × 10−5
M31 – centrifug viable supern 70° – 30 min	63
cells extracted in saline 70° – 30 min	1 2
extract	320
cells	2 0
M31 whole culture 70° – 30 min	1.3
supernatant	70
cells	

as centrifuged and the super
cells from this portion were
was inactivated by heat
or protective actin n

TABLE 4

Effects of Different Inactivation Procedures on the Toxicity and Immunogenicity of Salmonella typhimurium

Treatment		LD50 (mg)	LD50 (mg) 10−5
Whole culture	pressure	1 4	5 0
	γ irrad	0 9	1 3
	UV irrad	0 9	1.3
	60° C	0.9	1 0
	70° C	0 8	1 6
	100° C	1 4	5 0
	120° C	1 6	2 5
Cells	pressure	3 3	10
	γ irrad	1 6	40
	UV irrad	1 0	20
	60° C	1 6	10
	70° C	>4	70
	100° C	>4	>100
	120° C	>4	>100
Supernatant	pressure	1 1	1 6
	γ irrad	1 2	2 5
	UV irrad	1 2	1 3
	60° C	1 5	1 0
	70° C	1 0	1 2
	100° C	1 1	1 0
	120° C	0 9	2 0

Samples from a culture with a population density of 17 7 mg dry weight per ml were subjected to different treatments as indicated The high molecular weight materials released from the cells amounted to 5 10 per cent of the cell weight Pressure treatment was performed in a chamber described by *Heden* (1964) Complete inactivation was obtained when the cells were treated at 4000 atm for 10 minutes UV irradiation was achieved under two Philips TUV6 lamps at a distance of 10 cm for 30 minutes in a gently rocked petri dish Irradiation with γ rays was performed in a Co^{60} source with a dose rate of 10 000 rad min for 30 minutes These treatments were performed at room temperature

Our thanks are due to Prof *L. Ehrenberg* of the University of Stockholm *for permission to perform the γ irradiation in his department*

from four other cultures, the PD_{50} values were higher and varied considerably.

The next experiment showed that immunogenic substances are released from the cells during cultivation (Table 3). An extraction of immunogenic antigens takes place when cells are heat-treated at 70° for 30 minutes either in saline or in the growth medium. After the extraction cells have a much reduced immunogenicity.

The results in Table 4 are for an experiment where different methods of inactivating the cells were tested. During the different treatments, high molecular weight materials were released from the cells in amounts that permitted both dry weight determinations and animal tests to be performed. None of the procedures tested influenced the protective action significantly. Even treatment with 120° C for 15 minutes did not lower the immunizing power of the antigens released. The cells, however, retained little of their immunogenic substances after treatment at high temperatures.

TABLE 5

Protection Values Obtained from Control Groups during the Experimental Period

		PD_{50} (mg) $\times 10^{-5}$			PD_{50} (mg) $\times 10^{-5}$
Febr	24	10	May	16	10
	28	50		24	32
March	2	10	June	7	16
	14	25		28	16
	21	16	July	25	16
April	8	50	August	4	20
	13	16		10	25
	25	16			
	27	16			

TABLE 6

Protective Activity of Heat Killed Whole Cell Vaccines of Homologous and Heterologous Strains

	PD_{50} (mg)
Salmonella typhimurium 395M S form	50×10^{-5}
Salmonella typhimurium 395M R form	>0.2
Salmonella bonariensis (C_2 group)	>0.2
Escherichia coli B	>0.2

Table 5 is included to illustrate the reproducibility of the protection test during the experimental period and in Table 6 an experiment showing the specificity of the test is represented. Preparations of washed cell suspensions of heat killed bacteria were used as immunizing antigens. The antigen prepared with the homologous strains gave a PD_{50} within the range of the values obtained with the standard vaccine

Vaccines prepared from the R form and from two heterologous bacteria gave no protection even in doses 400 times the dose of the S form giving 50 per cent protection

DISCUSSION

The cell materials used in the present investigation were obtained by cultivation in a glucose-salts medium with effecient aeration and constant pH In a previous paper (*Holme & Edebo* 1961) it was shown, that cells grown in different media did not differ significantly in their virulence and toxicity The influence of cultivation conditions on the extractability of immunogenic components has, however, not been studied The bacteria used in the present study were very easily extracted by heat treatment in phosphate buffers or saline or in the culture medium This simple treatment has been used earlier for the extraction of endotoxin from *Echerichia coli* (*Roberts* 1949). Even during washing in the cold, immunogenic materials were released to such an extent that the localization of such materials to some structural component of the cell was very difficult

The toxicity and immunogenicity of the residue after extraction was generally very low It thus appears, that most of the immunogenic materials in heat-killed vaccines of *Salmonella typhimurium* is in the liquid portion Several traditional extraction methods using trichloroacetic acid, trypsin digestion, diethylene glycol and phenol-water mixtures were applied to the cells and the immunogenicity of the preparations determined (*Boivin et al* 1933, *Raistrick & Topley* 1934, *Morgan* 1937 and *Westphal & Luderitz* 1954) When applied to *Salmonella typhimurium* all these extracts gave a lower proctection level than the extracts obtained in saline with heat treatment

The mouse protection test appeared to be highly reproducible with the strain of mice used The reports in the litterature (*Hobson* 1957, *Baron & Formal* 1960), that killed vaccines do not give persistent protection could not be confirmed Cultivation tests for bacteria in the heart-blood and peritoneal fluid of mice surviving the protection tests did not give positive results Some of the groups were kept for two months after the termination of the proctection test and no further deaths were recorded At high doses of the immunizing antigen (≥ 0.1 mg) some deaths generally occurred within to or three days after the challenge was injected

SUMMARY

Cells and extracts of *Salmonella typhimurium* were tested for immunogenic activity and toxicity for mice Extraction of concentrated cell suspensions at 70° C for 30 minutes in saline resulted in active immunogenic preparations Comparable activities were found in the supernatant from heat-inactivated liquid cultures Antigens released

from four other cultures, the PD_{50} values were higher and varied considerably

The next experiment showed that immunogenic substances are released from the cells during cultivation (Table 3) An extraction of immunogenic antigens takes place when cells are heat-treated at 70° for 30 minutes either in saline or in the growth medium After the extraction cells have a much reduced immunogenicity

The results in Table 4 are for an experiment where different methods of inactivating the cells were tested During the different treatments, high molecular weight materials were released from the cells in amounts that permitted both dry weight determinations and animal tests to be performed None of the procedures tested influenced the protective action significantly Even treatment with 120° C for 15 minutes did not lower the immunizing power of the antigens released The cells, however, retained little of their immunogenic substances after treatment at high temperatures

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	25	16			
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TABLE 6

Protective Activity of Heat Killed Whole Cell Vaccines of Homologous and Heterologous Strains

	PD_{50} (mg)
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Escherichia coli B	>0 2

Table 5 is included to illustrate the reproducibility of the protection test during the experimental period and in Table 6 an experiment showing the specificity of the test is represented Preparations of washed cell suspensions of heat killed bacteria were used as immunizing antigens The antigen prepared with the homologous strains gave a PD_{50} within the range of the values obtained with the standard vaccine

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CULTIVATION OF SALMONELLA STRAINS IN CONTINUOUS CULTURE

By

T Holme and L Edebo

Received 28 vii 64

In an earlier communication from this laboratory equipment for growing aerobic bacteria in high yields has been described (*Edebo et al* 1962) Preliminary studies of the continuous propagation of *Salmonella typhimurium* in a similar apparatus have also been reported (*Holme & Edebo* 1961) Improvements in design, and further cultivation experiments, including continuous culture of different *Salmonella* species, are reported in this paper The simplicity of its operation makes the equipment suitable for the cultivation of bacterial cells for vaccine production

MATERIALS AND METHODS

Apparatus Design

The equipment was designed for a liquid volume of 0.5 litre The culture vessel was made of a *Pyrex glass tube closed* at one end The length of the tube was 450 mm and the internal diameter 60 mm The neck was closed by a rubber stopper perforated by glass tubes for air inlet for addition of fresh medium and alkali, and for inoculation Air was introduced at the bottom of the vessel through a small cylinder of fritted Pyrex gl[illegible]

w[illegible]

le[illegible]

by[illegible]

15[illegible]

fil[illegible]

Temperature control and control of pH were effected as previously described (*Edebo et al* 1962) In a few runs however, alkali was pumped into th[illegible] means of a [illegible]

which otherw[illegible]

The transfe[illegible]

was then effe[illegible]

the cotton pl[illegible]

described by [illegible] quality silicone tubing the pumps could be [illegible] been run for four weeks without breakage of the tubing

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Our thanks are due to Miss *Berit Lindholm* for her skilled technical assistance This work was aided by a grant from the Swedish State Medical Research Council

from cells inactivated by other treatments, i e high pressure, γ or UV-irradiation, did not show any higher immunogenic activity than the antigens released from heat-killed cells The toxicity and immunogenicity of the residual cell material after extraction was generally very low

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TABLE 1

Continuous Culture of Salmonella typhimurium 395M. A Glucose Salts Medium was Used with Glucose as the Limiting Factor. The Concentration of Glucose was 6 g per Litre and of Ammonium Chloride 3 g per Litre. Temperature 37° C. Dilution Rate 0.7 Hr⁻¹. Culture Volume 0.5 Litre

Day	pH	Dry weight of cells (g per litre)	Glucose (g per litre)
1	7.1	2.5	0
2	7.1	2.5	0
3	7.3	2.2	0
4	7.3	2.2	0
5	7.5	1.8	0
6	7.5	1.9	0
7	7.8	1.4	0
8	7.8	1.3	0
9	8.1	1.3	0
10	8.1	1.4	0
11	8.4	0.1	5.4
12	7.1	2.5	0
13	6.8	2.5	0
14	6.8	2.5	0
15	6.5	1.3	0

TABLE 2

Continuous Culture of Salmonella typhimurium 395M. A Glucose Salts Medium was Used with Ammonium Chloride as the Limiting Factor. The Concentration of Glucose was 20 g per Litre and of Ammonium Chloride 1 g per Litre. Dilution Rate 0.3 Hr⁻¹. Culture Volume 0.5 Litre. pH 6.8

Day	Temperature (°C)	Dry weight of cells (g per litre)	Glucose (g per litre)	NH_4^+ (g per litre)
1	37	1.7	4.9	0
2	37	1.6	5.1	0
3	33	1.6	5.9	0
4	33	1.6	5.9	0
5	29	1.5	7.3	0
6	29	1.5	6.4	0
7	25	1.4	8.7	0
8	25	1.5	7.2	0
9	35	1.5	7.0	0
10	39	1.4	6.5	0
11	39	1.5	6.7	0
12	37	1.6	6.3	0
13	37	1.5	6.1	0

39° C were applied at a dilution rate of 0.3 hr^{-1} in one glucose- and one nitrogen-limited culture. Steady-state growth was also obtained at pH values varying from 6.5 to 8.1. At a constant input concentration of glucose, in a glucose-limited culture, the highest population density was at pH 6.8 to 7.1. Dilution rates between 0.2 and 1.0 hr^{-1} were also applied. In these runs the population density was kept between 1.5 and 2.5 g dry weight of cells per litre of culture. At higher population densities, obtained by increasing the concentration of nu-

Operation

The culture vessel was sterilized empty After sterilization the pH electrode was fitted aseptically, and medium pumped into the vessel Inoculation was made with cells from a slant suspended in saline The culture was allowed to grow batchwise with aeration for about 8 hours before the continuous addition of fresh medium was started The dilution rate at the start was 0 3 hr^{-1} It was increased in three steps at 24 hour intervals to 0 7 hr^{-1} Higher dilution rates were usually not applied The cultures were aerated by an air flow of 0 5 l per minute Samples for analysis of dry weight of cells glucose and ammonium ions in the culture fluid were taken at the bottom of the culture through a needle perforating a rubber membrane (*Hedén & Holme* 1960)

The culture volume was kept constant by an overflow tube, through which the exit air also escaped From the receiving bottle the air was conducted to an empty bottle before entering a heated copper pipe for sterilization

Experimental

Strains The following strains were kindly supplied by the State Bacteriological Laboratory, Stockholm Sweden *Salmonella paratyphi A* var *durazzo* (IS 2), *S paratyphi BO* (IS 248), *S typhimurium* (395M), *S bonariensis* (IS 145) and *S typhi* (IS 58)

Media The strains *S paratyphi A S paratyphi BO* and *S typhimurium* grew on a
omposition glucose 10 g l NH_4Cl
2
g $MgSO_4$ 7 H_2O 0 2 g and 1 ml of
a
sulphate were sterilized separate
ly
before sterilization Acid produced
during the cultivation was neutralized by means of 2 M sodium hydroxide In a series of runs of the different *Salmonella* strains with the aim of testing the equip
plest was
l *S typhi*
per litre
nt strains
ne 1958)
owth *and*
biological activities of *S typhimurium* some modifications of the original medium were applied The concentrations of the carbon and nitrogen sources were varied in order to obtain different population densities and different limiting factors in the continuous cultures

Chemical techniques For dry weight determination of cells 25 ml samples were centrifuged at 10 000 × g the cells were suspended in 0 01 M phosphate buffer and again centrifuged at 10 000 × g in weighed glass tubes The cells were dried at 105° C overnight The glucose content of the supernatant was determined by the method given by *Hultman* (1959) and modified by *Rédei & Nagy* (1961) The indanetrione hydrate method was used for the ammonium ion determinations (*Jacobs* 1962)

Immunological techniques Mouse protection tests were performed as described elsewhere (*Holme & Edebo* 1965) Samples from the cultures were heat treated at 70° C for 30 minutes Four test samples and one control were analysed each time The control consisted of a heat killed suspension of cells obtained from a batch culture of the same bacterial strain The same control suspension was used during the whole experimental period It was kindly prepared by Dr H Billaudelle at the State Bacteriological Laboratory with the same techniques as used in standard vaccine preparation

RESULTS

The first series of experiments was designed to test the influence of temperature, pH and different limiting factors on growth and immunizing potency of *Salmonella typhimurium* cells Six runs of 15 to 20 days' duration were performed, three with glucose and three with ammonium chloride as the limiting factor Two of these experiments are presented in the Tables 1 and 2 Temperatures between 25° C and

mited by the nutrients in the medium, and the maximum density maintained with *Salmonella typhimurium* in a continuous run was 7 g dry weight per litre, corresponding to approximately 7×10^{10} bacteria per ml of culture The complication arising at higher population densities was increased foaming of the culture Higher densities were obtained in batch cultures using chemical defoamers (*Edebo et al* 1962) In the continuous cultures the dilution rate had to be decreased if defoamers were used to allow increased cell concentrations This could be due to oxygen deficiency Since the exhaust gas escaped through the overflow tube, and consequently the foam also, extensive foaming caused a decrease in the effective culture volume With the medium used, and with a population density of about 4 g dry weight of cells per litre, the foam layer on the top of the culture fluid did not exceed a depth of 10 mm

Before deciding on a certain process for the propagation of cells for vaccine production, detailed investigations are required for the evaluation of the optimal conditions for growth and immunogen production If possible the immunogenic substances should have low toxicity and be easily prepared from the cultural material The experiences from the present and earlier experiments do not indicate any definite advantage or disadvantage of continuous cultivation of *Salmonella typhimurium* for vaccine production as compared to the batch process as concerns the quality of the product The economics of the process is then the main factor to be considered This aspect will only be shortly discussed here, since it has been treated extensively by many other authors (*Herbert et al* 1956, *Maxon* 1955) The quantity of vaccine needed, the pre-existence of fermentation equipment for large-scale cultivat[illegible]

[illegible] represented by

s[illegible] litre of culture, a culture volume of 0 5 litre and a dilution rate of 0 5 hr^{-1} the output of cells would be 1 g dry weight per hour The vaccine dose for a human is 0 1 0 2 mg and the output corresponds consequently (if the higher figure is used) to 5000 doses per hour and 120,000 doses per day A batch culture with a working volume of 2 litres would produce the same amount in approximately the same time, assuming the population density obtained is 12 g per litre Since it is our experience that the product is of equal quality, irrespective of the cultivation method, only the need for large quantities of the vaccine should justify the use of continuous culture for the production of *Salmonella* vaccines

SUMMARY

A simple procedure for the continuous propagation of *Salmonella* cells for vaccine production is described Effective aeration and automatic pH control permitted the maintenance of a population density

trients in the medium, foaming became a problem It was noted, however, that at pH 6 8 less foam was formed than at pH 7 2 The highest bacterial concentration that could be maintained in a continuous run in the present equipment was 7 g dry weight of cells per litre of culture The maximum output was obtained at a dilution rate of 0 8 hr^{-1}

Forty samples, drawn from the continuous cultures and representing *different environmental conditions*, were tested for protection of mice to challenge with virulent *S typhimurium* None of the samples showed any significant deviation in immunizing potency as compared to the control suspension The PD_{50} was in no instance outside the values 10^{-4} and 10^{-7} mg dry weight of cells

A series of continuous cultures was also performed to show that the method could be adopted as a routine procedure in vaccine production *Salmonella paratyphi A* (I S 2), *S paratyphi BO* (I S 248), *S bonariensis* (I S 145) and *S typhi* (I S 58) were each grown continuously for three weeks in a medium consisting of the minimal medium enriched with casamino acids and yeast extract The nutrients were added in concentrations giving a yield of 4 g dry weight of cells per litre of culture The dilution rate was 0 5 hr^{-1} in these experiments

DISCUSSION

Continuous culture has been employed by a number of workers to grow cells for vaccine production *Salmonella typhi* (*Horodko et al* 1961), *Brucella abortus* (*Hauschild & Pivnick* 1961) and *Pasteurella pestis* (*Pirt et al* 1961) have been successfully propogated by this method There are some properties of a continuous process which makes it attractive for vaccine production Large quantities can be produced in comparatively small vessels and a more homogeneous product is obtained than in a corresponding batch process However the most important advantage of a continuous culture method is that it offers greatly increased scope for studying the influence of environmental conditions *on the formation of immunogenic substances in the* bacterial cells In a batch process the concentration of nutrients changes, and metabolites accumulate, during the growth of the culture In a continuous culture a steady state can be maintained for a considerable period of time

The selection of avirulent non-immunogenic mutants in continuous culture has been observed in studies of *Brucella abortus* and *Pasteurella pestis* In both instances this selection could be counteracted by a careful adjustment of the cultural conditions In continuous cultures of the different *Salmonella* species *no changes in colonial morphology* indicating the existence of avirulent mutants in measurable quantities were observed, nor did the biological tests with *Salmonella typhimurium* indicate any decrease in immunogenicity in continuous cultures

The population density in the present apparatus was purposely li

mited by the nutrients in the medium and the maximum density maintained with *Salmonella typhimurium* in a continuous run was 7 g dry weight per litre corresponding to approximately 7 × 10¹⁰ bacteria per ml of culture. The complication arising at higher population densities was increased foaming of the culture. Higher densities were obtained in batch cultures using chemical defoamers (*Edebo et al.* 1962). In the continuous cultures the dilution rate had to be decreased if defoamers were used to allow increased cell concentrations. This could be due to oxygen deficiency. Since the exhaust gas escaped through the overflow tube and consequently the foam also, extensive foaming caused a decrease in the effective culture volume. With the medium used and with a population density of about 4 g dry weight of cells per litre the foam layer on the top of the culture fluid did not exceed a depth of 10 mm.

Before deciding on a certain process for the propagation of cells for vaccine production, detailed investigations are required for the evaluation of the optimal conditions for growth and immunogen production. If possible the immunogenic substances should have low toxicity and be easily prepared from the cultural material. The experiences from the present and earlier experiments do not indicate any definite advantage or disadvantage of continuous cultivation of *Salmonella typhimurium* for vaccine production as compared to the batch process as concerns the quality of the product. The economics of the process is then the main factor to be considered. This aspect will only be shortly discussed here since it has been treated extensively by many other authors (*Herbert et al.* 1956, *Maxon* 1955). The quantity of vaccine needed, the pre-existence of fermentation equipment for large scale cultivation and many other factors must be considered.

The capacity of the equipment described here can be represented by some average figures. At a population density of 4 g per litre of culture, a culture volume of 0.5 litre and a dilution rate of 0.5 hr^{-1} the output of cells would be 1 g dry weight per hour. The vaccine dose for a human is 0.1–0.2 mg and the output corresponds consequently (if the higher figure is used) to 5000 doses per hour and 120 000 doses per day. A batch culture with a working volume of 2 litres would produce the same amount in approximately the same time assuming the population density obtained is 12 g per litre. Since it is our experience that the product is of equal quality irrespective of the cultivation method, only the need for large quantities of the vaccine should justify the use of continuous culture for the production of *Salmonella* vaccines.

SUMMARY

A simple procedure for the continuous propagation of *Salmonella* cells for vaccine production is described. Effective aeration and automatic pH control permitted the maintenance of a population density

of 7 g dry weight per litre of culture in a glucose-salts medium As standard practice a complex medium, limiting the yield to 4 g dry weight per litre, is recommended Five different strains, representing the serological groups A, B, C and D have been grown for three week periods No significant variations in immunogenicity for mice were observed in vaccines prepared from *Salmonella typhimurium* grown under different environmental conditions

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IN OVO PRODUCTION OF INTERFERON INDUCED BY INFLUENZA VIRUS OF VARYING DEGREES OF INCOMPLETENESS

By

F. A. HAHNEMANN and V. REINICKE

Received 20 viii 64

The experiments by *Isaacs & Lindenmann* (9, 10) showed that infection of chorioallantoic membranes in vitro with influenza virus could induce the production of a virus inhibiting substance, which was named interferon (2, 11, 15) Their observations seemed to indicate that only virus particles which had been damaged by partial inactivation or had been made incomplete by undiluted passages, were able to induce the production of interferon (1, 11) Since then, however, it has been repeatedly established (2, 4, 5, 7, 8, 12, 19, 20 and others) that interferon may be produced also by cells infected with fully active virus *Burke & Isaacs* (2) have suggested that under such conditions the interferon production might likewise be due to incomplete or heat inactivated virus which had accumulated during the preceding hours of infection

The purpose of the present study was to examine the relationship between production of incomplete virus and interferon in a series of undiluted egg passages, in particular the ability of influenza virus of various degrees of incompleteness to stimulate the production of interferon in ovo

MATERIALS AND METHODS

Virus

Influenza B Lee virus in its 7th egg passage (Titre $10^{9.?}$ FID_{50} per ml) in this laborat ry was employed in the pres nt st d D
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The auth rs are indebted to Dr *P von Magnus* for his interest in this work They also wish to thank Mrs *A Diemer* for skilled technical assistance

Influenza A Melbourne in its 5th egg passage (Titre $10^{9.6}$ *EID$_{50}$ per ml) in this laboratory* was employed as challenge virus in assays of interferon and for production of ultraviolet (UV) inactivated virus

Eggs Nine to thirteen day-old white Leghorn eggs were employed

Haemagglutinin (HA)- and Infectivity titrations Freshly harvested pools of allantoic fluids were titrated both for haemagglutinin and for infectivity Samples for re titrations were kept at +4° C and —60° C respectively HA titrations were *performed according to the method of Salk* (18) Infectivity titrations were carried

ly old chick embryos with 0 2 ml amounts

virus Phosphate buffer at pH 7 38 con

diluent After 64 hours of incubation the eggs were harvested individually and tested for haemagglutinins and the 50 per cent infectivity end point (EID$_{50}$) was calculated according to the method of *Karber (14)* The titres are expressed as the number of EID$_{50}$ or haemagglutinating units present in 1 ml of the undiluted fluids

Antibiotics G penicillin and streptomycin were added to allantoic fluids and diluents in sufficient amounts to give a final concentration of 200 units/ml and 50 γ/ml respectively

Interferon assay Before measurement of the content of interferon took place interfering virus was removed from the allantoic fluids or inactivated by one of the following methods

1 Partial removal of the virus by haemadsorption with a 10 per cent suspension of chick erythrocytes for one hour at +4° C followed by ultracentrifugation in a Spinco Model L centrifuge for two consecutive one hour cycles at 105 000 g and 144 000 g respectively

2 Heating to 65° C for one hour

3 Dialysis against a glycine buffer at pH 2 0 for 24 hours followed by repeated dialysis against Earle s BSS until a pH of approximately 7 0 was restored The dialysis was carried out at +4° C under continuous rotation of cellulose dialysis casings obtained from Kalle Aktiengesellschaft Wiesbaden Biebrich Germany

It has been shown (2 21) that each of these procedures is able to remove or destroy the interfering activity of influenza virus without any significant damage to the interferon As a control on the removal respectively the inactivation of the virus HA titrations and infectivity titrations were carried out

The interferon assay

Serial 4 fold dilutions ere incubated in 1 ml amounts in roller tubes chorioallantoic membrane These membrane from 11 or 12 day old chick embryos Six roller tubes were employed per dilution and the membranes were incubated in a roller drum at 37° C for 24 hours Membrane pieces incubated in a roller tube with inactivated allantoic fluid from 13 day old non inoculated eggs served as control

Subsequently the dilutions of allantoic fluids were removed and replaced with 1 ml of a 10^{-3} dilution of an influenza A Melbourne stock having a titre of 10^{9} EID$_{50}$ per ml After additional 48 hours of incubation the fluids were pooled according to groups and titrated for haemagglutinin

The titre ($\log_{10}$) of interferon was calculated according to the method of Karber and expressed as the dilution of allantoic fluid which reduced the production of haemagglutinin 50 per cent in comparison to the controls

EXPERIMENTAL

The experiments described in the following were all performed by inoculation of influenza B Lee virus into the allantoic cavity of 9–13-day-old embryonated eggs The eggs were incubated at 35° C and experiments were timed in such a way, that all eggs were 13 days old at the time of harvest

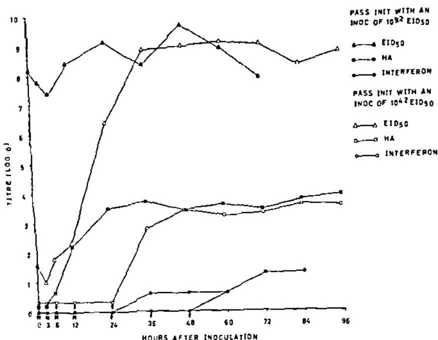


Fig 1

Virus and Interferon Titres of Allantoic Fluids from two Egg Passages of Influenza B Lee Virus Initiated with an inoculum of $10^{9.2}$ EID$_{50}$ and $10^{4.2}$ EID$_{50}$ Respectively

Interferon Production Following Inoculation of Undiluted and 10^{-5} Diluted B Lee Stock Virus

9 13 day old eggs were inoculated with a freshly harvested standard passage of influenza B Lee virus which had a titre of $10^{9.2}$ EID$_{50}$ per ml One half of the eggs were inoculated with 0 5 ml amounts of the undiluted standard passage fluid, the other half with 0 2 ml amounts of a 10^{-5} dilution At intervals, the allantoic fluids from 25 eggs of each group were harvested, pooled and titrated for haemagglutinin and infectivity Aliquots of the fluids were dialysed against a buffer at pH 2 to destroy the virus and subsequently tested for interferon The results are shown in Fig 1 As can be seen, measurable amounts of interferon appeared between 12 and 24 hours of incubation with the undiluted inoculum and after 48–60 hours of incubation with the 10^{-5} diluted seed The final level of interferon, however, seemed to be the same irrespective of the size of inoculum

Interferon Production in Undiluted Passages of Influenza B Lee Virus

A freshly harvested influenza B Lee standard passage (containing $10^{9.0}$ EID$_{50}$ per ml) and seven consecutive undiluted passages (UP 1 to UP 7) from this stock were inoculated in 0 5 ml amounts by the

INOCULA	ST	UP 1	UP 2	UP 3	UP 4	UP 5	UP 6	UP 7
EID_{50}	9.0	9.6	9.4	9.8	7.8	5.8	6.4	3.8
HA	3.18	3.33	3.33	3.33	3.26	2.58	1.05	<0.30
EID_{50}/HA	5.82	6.27	6.07	6.47	4.54	3.22	5.35	>3.80

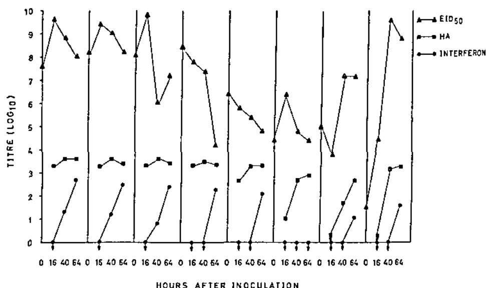


Fig 2

Virus and Interferon Titres of Allantoic Fluids in Egg Passages Initiated with Undiluted Allantoic Fluid Inocula Obtained from Serial Passage of Undiluted Influenza B Lee Virus

allantoic route into 10–12-day old eggs The allantoic fluids were harvested and pooled from groups of 40 eggs after 16, 40 and 64 hours of incubation and titrated for infectivity and haemagglutinin The virus was inactivated in aliquots of these fluids either by combined haemadsorption and ultracentrifugation or by acid inactivation (see Materials and Methods) and the inactivated fluids were titrated for interferon The results are recorded in Fig 2 from which it can be seen that with increasing number of passages, the appearance of interferon was increasingly delayed, and after 5 undiluted passages no interferon was demonstrable In the following two passages interferon reappeared in increasing amounts

Undiluted seeds of increasing incompleteness thus gave rise to yields containing decreasing amounts of interferon In a subsequent experiment the effect of dilution of these incomplete seeds was studied The standard passage virus and the first 4 undiluted egg passages were inoculated in a 10^{-5} dilution into 9–12-day-old eggs, and the allantoic fluids from groups of 20 eggs were harvested and pooled after 16, 40, 64 and 88 hours of incubation After acid inactivation the content of interferon was measured and found to be almost the same in all five passages (Fig 3)

In ovo production of interferon with seed virus partially inactivated

INOCULA	ST	UP 1	UP 2	UP 3	UP-4
EID_{50}	8 4	9 2	8 6	7 2	5 6
HA	3 3 3	3 0 3	2 9 6	3 0 3	3 11
EID_{50}/HA	5 0 7	6 1 7	5 6 4	4 1 7	2 4 9

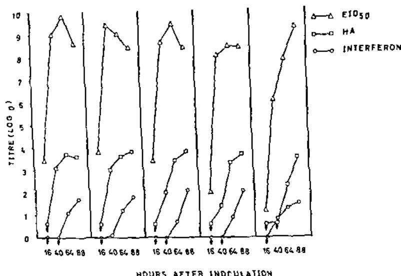


Fig 3

Virus and Interferon Titres of Allantoic Fluids in Egg Passages Initiated with 10^{-6} Diluted Allantoic Fluid Inocula Obtained from Serial Passage of Undiluted Influenza B Lee Virus

by ultraviolet light was investigated in a separate experiment A freshly harvested standard passage of influenza A Melbourne virus was purified by haemadsorption with 4 per cent chick erythrocytes for one hour at 0° C followed by elution into a phosphate buffer (containing 0 001 per cent $CaCl_2$) for four hours at 37° C Aliquots of this virus suspension were inactivated by ultraviolet irradiation by a Philips germicidal lamp for different periods of time The UV inactivated virus suspensions were inoculated in 0 5 ml amounts into groups of ten 12 and 10-day old eggs which were harvested after 16 and 64 hours of incubation respectively After heat inactivation of the virus the content of interferon in the pools of the allantoic fluids was measured The result appears from Fig 4 which shows that the production of interferon decreased when the infectivity of the inoculum decreased and that no production of interferon could be demonstrated when the infectivity of the inoculum had been destroyed

UV INACT (SEC)	0	10	20	30	60
EID_{50}	9 6	5 4	2 0	0 4	0 0
HA	4 16	4 24	4 24	4 24	4 24

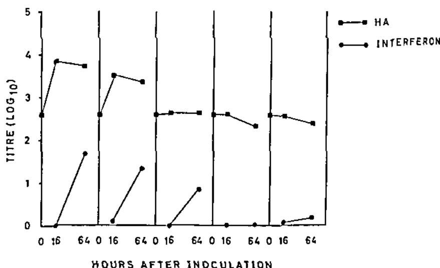


Fig 5

Interferon and Haemagglutinin Titres of Allantoic Fluids from Eggs Inoculated with UV Inactivated Influenza A Melbourne Virus

DISCUSSION

In the present experiments the production of interferon in undiluted passages of influenza B Lee virus has been followed An attempt has been made to analyse the relation, regarding quantity as well as chronology between the production of the three components interferon, infectious virus and incomplete virus

The experiments have shown that inocula consisting of standard virus or of the first undiluted passages which contain a relatively high amount of complete virus induced a stronger and earlier production of interferon than did the following undiluted passages consisting mainly of incomplete virus The delayed and diminished production of interferon was most marked in the eggs inoculated with UP 5, i e with the inoculum containing the highest amount of incomplete virus (EID_{50}/HA ratio 3 2 (See Fig 2))

The present study has on the other hand not shown any clear quantitative correlation between the production of interferon and the amount of inoculated infectious virus or the amount of infectious virus produced in the eggs Undoubtedly the production of interferon decreases from passage to passage, but while the infectivity titre shows a 100 to a 1000 fold decline through the first five passages, the reduction of the final titre of interferon is only around 4-fold (experiment 2 (Fig 2)) Very similar results can be seen in experiment 4

(Fig 4) which shows decreasing production of interferon by inocula consisting of influenza virus of increasing degrees of UV inactivation

It has not been possible either to show any clear correlation between the production of interferon and the amount of inoculated haemagglutinin or the amount of haemagglutinin produced in the eggs Experiment 2 (Fig 2) shows a considerable production of haemagglutinin without any detectable production of interferon following inoculation of UP 5 virus The increasing production of interferon after inoculation of UP 6 and UP 7 is most likely due to the relatively low amount of virus in the inocula so that these undiluted passages more or less are comparable to standard passages

The results obtained in the present studies on the production of interferon following inoculation of complete and incomplete virus differ to some extent from observations by Isaacs et al (1 2 12 15) on the production of interferon in chorioallantoic membranes The discrepancies may possibly be due to the different experimental conditions

The question whether interferon plays a role in the formation of incomplete virus (6 17) must also be answered in the negative The fluids employed as seed for the UP passages were in all instances harvested after 16 hours incubation of the eggs at a time where undoubtedly only small and successively decreasing amounts of interferon were present in the fluids From experiment 2 (Fig 2) can be seen that no production of interferon could be detected during the entire experimental period after inoculation of UP 5 It can also be seen that UP 5 seed causes the most marked production of incomplete virus Consequently the passage inoculated with UP 5 seed demonstrates that an inoculum containing a low amount of interferon may cause a marked production of incomplete virus and no detectable production of interferon The decreased production of virus after inoculation of UP 6 is most likely also without correlation to the production of interferon since high yields of virus as well as interferon can be obtained simultaneously as appears from the passages inoculated with UP 1 and UP 2

It seems therefore unlikely that the production of incomplete virus in undiluted passages of influenza B Lee virus can be ascribed to an effect of interferon

This conclusion is in accordance with the results obtained in another study on the same problem (17)

SUMMARY

In a series of experiments the ability of incomplete influenza B Lee virus to induce the production of interferon in ovo was investigated It was shown that the production of interferon decreased simultaneously with increasing incompleteness of the virus inoculum Essentially

similar results were obtained when inocula consisting of ultraviolet inactivated influenza A Melbourne virus were employed

It was furthermore shown that virus inocula which induced production of only a small amount of interferon were superior in inducing production of virus of a high degree of incompleteness This observation is not compatible with the idea that production of interferon is a necessity for the production of incomplete influenza virus

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VIRAL SUSCEPTIBILITY AND EMBRYONIC DIFFERENTIATION

6 *Cytochemical Studies on Normal and Polyoma Infected Foetal Kidney in vitro*

By

JUHANI RAPOLA, LAURI SAXÉN and TAPANI VAINIO

Received 28 viii 64

Embryonic tissues have been accorded special attention in studies on changes in viral susceptibility *Chapronière* (1957) noted that cell cultures of rat embryos and newborn rats lost their ability to support myxoma virus replication, together with an increasing differentiation rate of the tissues Similar results, which indicate alteration and general diminution of the viral susceptibility of embryonic cells when the tissues reach a higher stage of differentiation, have been reported by a number of authors (reviewed by *Ebert & Wilt* 1960, *Saxén* 1964)

The need for a suitable experimental model applicable to studies of

[illegible] differentiate *in vitro* (*Saxén et al* 1962) The metanephrogenic mesenchyme has been shown to be susceptible to polyoma virus, to judge from the histopathological lesions (*Saxen et al* 1963), the synthesis of viral antigen (*Vainio et al* 1963 a b) and the presence of virus like particles (*Wartiovaara et al* 1964) When the metanephrogenic mesenchyme becomes involved in tubulogenesis [illegible]

[illegible] components of developing mouse kidneys suggests the need for an analysis of the metabolism of these tissue components Previous reports have indicated that there are differences in the activities of certain enzymes between the tubular and mesenchymal components Additionally, som alterations in the activities of

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[illegible] wish to thank Mrs Anja Tuomi Miss Ann Kristin Thors and Miss Liisa Helleman for technical assistance

various enzymes have been shown to be related to the early steps of tubulogenesis (*Rapola et al* 1963a,b, *Rapola & Niemi* 1964) In the experiments described here, we used the cytochemical demonstration of the activities of different enzymes to analyse the differences between the tissue components in our model, and to study the effect of infection with polyoma virus on the activities of these enzymes

MATERIAL AND METHODS

Cultures and virus infection The kidneys of 1
mice embryos were removed and cultivated in a .
of the cultures were infected with SE polyom
remainder served as controls Details of the methods of culture and of infection have been given in previous papers (*Saxen et al* 1962 1963) In experiments designed to demonstrate tetracycline fluorescence alkaline phosphatase and succinic tetrazolium reductase kidneys cultivated for 4 to 9 days were used Observations with regard to the activities of the remainder of the enzymes were derived from the explants cultivated for 6 to 9 days For the demonstration of each enzyme or tetracyclin fluorescence three or four explants were used on each occasion, and the series duplicated

Tetracycline and alkaline phosphatase 24 hours before collecting the cultures purified tetracycline ("Tetracyn", Pfizer) was added to the culture medium at a final concentration of 40 μg/cc (*Saxen & Vainio* 1964) The infected and intact explants were removed from the supporting Millipore filters, embedded in fresh mouse liver tissue, and frozen sections were prepared in a cryostat The sections were picked on microscopic slides and post fixed in cold (4° C) absolute acetone for 1 hour The unstained sections were examined and photographed under UV light Subsequently the sections were stained with haemalum eosin or assayed for the alkaline phosphatase by means of *Gomori's* Ca Co method (1952), glycerophosphate serving as substrate Control experiments showed that neither tetracycline treatment nor previous UV photograp f the
unspecific alkaline
As a consequence
phatase and tetracycline incorporation in the same tissue section

Succinic tetrazolium reductase The kidney explants were embedded in fresh mouse liver and unfixed frozen sections treated according to *Pearse* (1960), with Nitro BT as the final electron acceptor

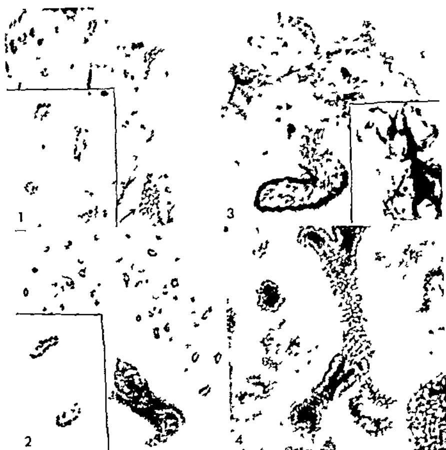
Other phosphatases For the demonstration of acid phosphatase (AP ase) adenosine triphosphatase (ATP ase) and thiamine pyrophosphatase (TPP ase) the kidneys were fixed in 4 per cent formalin buffered to neutrality at 4° C for 12 hours After fixation they were embedded in 10 per cent gelatine and frozen sections prepared in a cryostat *Gomori's* (1952) method for AP ase *Wachstein & Meisel's* (1957) method for ATP ase and *Novikoff & Goldfisher's* (1961) method for TPP ase were employed

Time lapse cinematography For this purpose the kidneys were cultivated in Rose chambers under a film of reconstituted rat tail collagen and exposures taken every third minute for up to 14 days Analysis of the films was carried out in a motion analysis projector MK 2 (*Saxen & Korhonen* 1964)

RESULTS

Uninfected Foetal Kidneys in vitro

As has been described previously (*Saxen et al* 1962, 1963), under the conditions used the kidneys develop by forming branches of collecting tubes as derivatives of the ureteric bud and primordia of secretory tubules, which differentiate from the metanephrogenic mesenchyme There was no marked change in the pattern of the cytochemically demonstrable enzymes during the 6 to 9 days of cultivation The follow-



Figs 1 4

Fig 1 The distribution of acid phosphatase in a seven day old kidney explant High activity in the ureteral mesenchyme is indicate l by an arrow In the left hand corner at higher magnification tl ere can be seen the granular reaction product in the tubules and occasional granules in the mesenchyme × 100 deta l × 250

F g 2 TPP ase in a seven day old explant The d stribution of the enzyme activity is very similar to that in I ig 1 but the reaction product is coarser and even lamellar as can l e remarke l in the detail p cture in the left han l corner × 100 deta l × 250

F g 3 ATP ase in a seven day old kidney explant Activity is ol servable in the tubular portions and surrounding the ureter rudiment In the detailed pic ture in the right hand corner is a cross section of a tul ule The highest activity is between the tul ular cells and in the luminal cytoplasm × 100 detail × 1000

Fig 4 Succinic tetrazolium reductase in a seven day old explant The activity is concentrated in the tubules in the apical cytoplasm not only the diffuse staining but also some particulate staining can be seen The mesenchyme l etween tubular portions has very l ttle formazan staining and is gathered on the lip d granules × 360

ing account is based on the explants cultivated for seven days, a time when the lesions in the infected explants are widely distributed and intense

Stromal mesenchyme The only enzyme activity of noticeable intensity was that of alkaline phosphatase (Fig 9b) A few granules which displayed acid phosphatase were seen occasionally in the mesenchyme (Fig 1) The activity of succinic tetrazolium reductase was very faint, the reaction product being inclined to gather on the lipid granules abundant in the mesenchyme No activity was observed as concerns ATP-ase or TPP-ase Only an occasional cell showed tetracycline fluorescence

Tubular parts of the explants Both the collecting tubes and the secretory tubules exhibited a granular reaction product in the apical cytoplasm of the tubulus cells when incubated for AP-ase (Fig 1) Particulate, somewhat elongated, even lamellar, particles displaying enzyme activity were discernible in all epithelial components of the kidneys after TPP-ase assay (Fig 2) The branches of the collecting system, and at least part of the secretory tubules also had ATP-ase activity The enzyme activity was concentrated in the luminal cytoplasm and luminal cell borders, and in the intercellular borders of the tubules (Fig 3) Both the secretory tubules and the collecting tubes

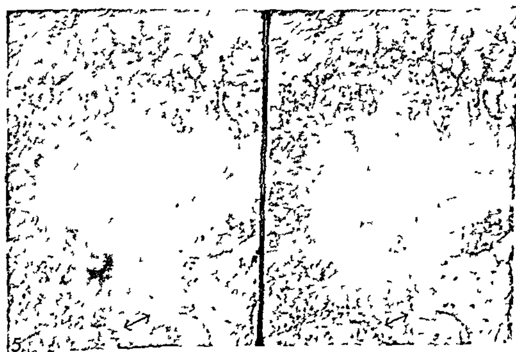


Fig 5

Two subsequent pictures from the time lapse film The tight ureteral mesenchyme is seen surrounding the ureter Note the changes in the contours of the ureter The lines drawn across the inner diameter of the ureter show the method used in the pulse analysis presented in Fig 6 × 150

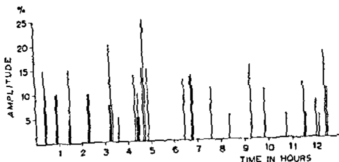


Fig 6

The amplitude of the pulse in the ureter over a period of 12 hours The cross diameter of the ureter is measured as depicted in Fig 5 and the zero line on the longitudinal axis represents the 'resting' phase when no pulses are discernible The pictures were taken every third minute

showed activity as regards succinic tetrazolium reductase Very intense staining both diffuse and particulate, was observable in the luminal cytoplasm (Fig 4)

Neither alkaline phosphatase activity nor tetracycline fluorescence could be seen in the epithelial elements of the explants

Ureteric mesenchyme The short unbranched ureter rudiment was surrounded by a tight mesenchyme, which from a histological point of view was very similar to the mesenchyme elsewhere in the explants While the enzymo morphology of the ureteric epithelium did not differ considerably from that of the collecting tubes, the mesenchyme had some characteristic features which distinguished it from the rest of the mesenchyme It exhibited the highest phosphatase activity of all substrates used in these experiments (Figs 1, 2, 3 and 9b)

Additional knowledge of the character of the ureteric mesenchyme was derived from time lapse cinematographs After 6 to 7 days of cultivation, irregular pulsations occurred in the mesenchyme In Fig 5, two subsequent phases of the ureteral pulses are represented, and in Fig 6 a calculation of the frequency and the amplitude of the circular contractions In addition to these contractions, pulses longitudinal to the axis of the ureter were seen

The Kidneys after Polyoma Virus Infection

Bright yellow tetracycline fluorescence was remarked on the sixth day of cultivation The maximum fluorescence was noted on the 7th and or 8th day of cultivation, this remained unchanged until the 9th day It was regularly located in the mesenchymal parts of the kidneys Not only the mesenchyme between the collecting tubes and secretory tubules, but also the ureteric mesenchyme had tetracycline fluorescence (Figs 7 and 8) Only on occasion did the epithelium of the kidney tubules and uretic bud show some single fluorescent cell

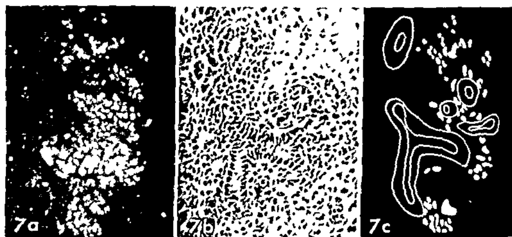


Fig 7

Picture *a* depicts tetracycline fluorescence on the 6th day after polyoma infection The same section after haemalum eosin staining is illustrated in picture *b* Picture *c* was obtained by superimposing the negatives for pictures a and b and by subsequent drawing of the tubules and fluorescent parts × 250

The areas which displayed tetracycline fluorescence and alkaline phosphatase seemed to be correlated, although the distribution of fluorescence was wider than the areas where alkaline phosphatase activity was observable (Fig 9)

On the 7th, and especially on the 8th day of cultivation, (*i e* on the 6th and 7th days after virus inoculation) it became evident that the



Fig 8

Pictures *a* and *b* are from the area of the ureter where the tetracycline fluorescence (a) and subsequent normal histology (b) can be seen × 250

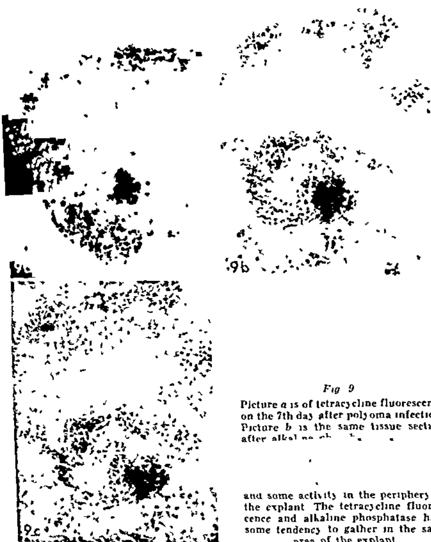


Fig 9

Picture *a* is of tetracycline fluorescence on the 7th day after polyoma infection Picture *b* is the same tissue section after alkal ne ph ...

and some activity in the periphery of the explant The tetracycline fluorescence and alkaline phosphatase have some tendency to gather in the same area of the explant

activity of the AP ase had increased in the mesenchyme of the explants This was seen as a rise in the number and the size of the AP-ase positive particles (Figs 10 and 11) The infection exercised no noticeable effect upon either the activity or the distribution of the tubular AP-ase Neither did it affect the activities of the ATP-ase, TPP-ase and alkaline phosphatase

tet

de

10

11

Figs 10 11

Fig 10 Distribution of the acid phosphatase in the periphery of an explant after 7 days of polyoma infection The coarse reaction product is dispersed in the mesenchyme between tubular parts × 100

Fig 11 A cross section of the ureter of a 7 day infected kidney Very high acid phosphatase activity is discernible in the mesenchyme surrounding the ureter × 250

DISCUSSION

In previous studies using the same developing model and the same virus, much information has been gained on the susceptible and resistant tissue components and their relations to tubule differentiation (*Saxén et al* 1962, 1963, *Vainio et al* 1963a b,c) The methods applied in this study permit of the addition of new characteristics as regards the resistant and susceptible tissue components

It has been postulated that, with the methods employed for demonstration of the activities of AP-ase, ATP ase and TPP-ase, they exhibit some association with the known cytoplasmic organelles (*Novikoff & Essner* 1962) Thus they can be used as markers for the lysosomes and related particles, for plasma and cell membranes, and for the Golgi apparatus respectively Succinic tetrazolium reductase activity, and especially the particulate reaction product, can be given tentative consideration as markers for mitochondrial enzyme activity (*Pearse* 1958) The activities of these enzymes appeared to bear a distinct similarity to features known about the tubules of the adult kidneys (*Novikoff* 1960), and represent an advanced stage of differentiation Against this, it is known that both the resistance to polyoma virus (*Vainio et al* 1963b and c) and the integration of the enzyme activities mentioned (*Rapola & Niemi* 1964) begin in the very early stage of tubular differentiation

The enzymatic pattern of the mesenchyme susceptible to virus, the ureteric mesenchyme exclusive, was very poor, and in striking contrast to the well organized tubules with activity which extends to a number of enzymes Non specific alkaline phosphatase was the only active enzyme detected in this mesenchyme It is known that alkaline phosphatase is widely distributed in the undifferentiated tissues, and in tissues which are undergoing morphogenesis in embryos However, the activity of the alkaline phosphatase usually diminishes when the tissues attain a certain degree of morphologically distinct differentiation (for references see *Rapola et al* 1963b)

It has been reported in a number of studies that the viral lesions are accompanied and in some cases preceded by changes in the enzyme activities of the host cells cultivated *in vitro* (*Kovacs* 1956, *Bachtold et al* 1959 *Allison & Sandelin* 1963 *Fortelius* 1963 *Godman et al* 1964) Nevertheless the observations have varied somewhat, and one can hardly as yet discover general rules of the relations of the viral cytopathic effects (CPE) and enzymatic changes in host cells The only change at the cytochemical level in the enzyme activities studied, was the increase in activity of the AP ase in the mesenchyme This observation might be related to findings made by *Allison & Burston* (1964), who noted a such change in liver lesions as a result of murine hepatitis virus and it may support the idea of the role of lysosomes in damaged tissues (*De Duve* 1963) So far, however, we have failed to find





Figs 10 11

Fig 10 Distribution of the acid phosphatase in the periphery of an explant after 7 days of polyoma infection The coarse reaction product is dispersed in the mesenchyme between tubular parts × 100

Fig 11 A cross section of the ureter of a 7 day infected kidney Very high acid phosphatase activity is discernible in the mesenchyme surrounding the ureter × 250

enchyme but not in the epithelium At the same time as the susceptible tissue exhibits a viral cytopathic effect, activation of acid phosphatase can be shown

The ureteric rudiment of the kidney explants is surrounded by tightly packed mesenchymal cells These cells differentiate to a contractile tissue as seen in time lapse cinematography In addition to the contractions visible in cinematography this mesenchyme differs from the stromal mesenchyme by possessing a very high phosphatase activity with all substrates used Although this tissue can be considered as differentiated tissue like the epithelium, and although it has in part the same enzyme activities as the epithelium, it still remains susceptible to polyoma virus like the stromal mesenchyme

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8 *Godm* [illegible]

9 *Gom* [illegible]

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16 [illegible]

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an early histochemical marker for viral CPE in our experimental model The above change in the enzyme activity does not precede the tetracycline incorporation used as a marker in our previous studies (*Saxen & Vainio* 1964).

In our discussion of the mechanism of the acquisition of virus resistance in maturing tissues, the term "viral competence" has been suggested (*Saxen et al* 1963, *Saxen* 1964) The phenomenon has been compared with well-known examples in embryogenesis where differentiating cells lose their capacity, the "competence" to respond to environmental, morphogenetic stimuli (cf *Saxen & Toivonen* 1962) In other words, differentiation is followed by a decreased susceptibility to different epigenetic stimuli, and the virus might in fact be one of those Nevertheless, the present results seem to present one exception to this working hypothesis. Both cytochemical studies and observations in time lapse cinematography have indicated the receipt by the ureteric mesenchyme in our model of some special properties during the course of development However, this has not led to viral resistance being observable in the tubular epithelium originally derived from the same susceptible progenitor cells, but differentiated into another direction

Finally, the observations made on the cytochemistry and virus susceptibility of the different tissue components in our model have taught us another general limitation of such studies The acquisition of virus resistance during tubulogenesis could be correlated to certain simultaneous changes in the enzyme activity of the differentiating cells, but a very similar change in the enzymatic pattern during development of the ureteric mesenchyme obviously had no bearing on virus resistance Thus, once again, we are obviously dealing with parallel but not necessarily related phenomena during embryonic differentiation

SUMMARY

The distribution patterns of alkaline and acid phosphatase, adenosine triphosphatase, thiamine pyrophosphatase and succinic tetrazolium reductase have been studied in developing mouse kidney rudiments, *in vitro*, and in similar explants infected with polyoma virus In the infected explants, viral lesions were demonstrated with tetracycline fluorescence

Most of the enzymes examined have been demonstrated to be active in the kidney epithelium, which is resistant to polyoma virus The topography of these enzymes at cellular level is also well defined However, alkaline phosphatase activity is lacking in the epithelium On the other hand, the stromal mesenchyme, which is highly susceptible to infection with polyoma virus, *exhibits alkaline phosphatase activity only*, the other enzymes tested being not demonstrable

During polyoma infection tetracycline is incorporated in the mes-

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MONOPHASISCHE SALMONELLA-KULTUREN ZUR H-SERUM-HERSTELLUNG

Von

F KAUFFMANN

Eingegangen 18 IX 64

Nachdem bereits im Jahre 1946 einige, monophasische *Salmonella*-Kulturen von *Edwards & Bruner* zur Herstellung diagnostischer Seren beschrieben wurden, sind im Laufe der letzten 20 Jahre uber 100 monophasische Kulturen bekannt geworden Hierbei sind aber die häufigen, monophasischen *species*, die den G-Komplex besitzen, nicht mitgerechnet In der folgenden Tabelle sind also nicht alle bekannten, monophasischen Kulturen enthalten, sondern nur solche, die zur Herstellung monophasischer H-Seren von praktischer Bedeutung sind

In der Diagnostik der *Salmonella-species* spielen nämlich monophasische H-Seren eine grosse Rolle, da sie erstens für die Objektglas-Agglutination und zweitens zur Herstellung von *Gard*-Platten verwendet werden Besitzt man derartige, monophasischen H-Seren, so benotigt man keine Absorptionen, und zwar weder die Entfernung von H-Antikorpern der anderen Phase noch die Beseitigung von O-Antikorpern Wurden die Seren mit rein monophasischen Kulturen hergestellt, so enthalten sie keine Spur von H-Antikorpern der anderen Phase Naturlich besitzen derartige Seren ausser den H-Antikorpern der eigenen Phase noch die entsprechenden O-Antikorper, doch kann man sich dadurch helfen, dass man je nach Bedarf ein H-Serum, das zu einer anderen O-Gruppe gehört, verwendet

Es ist deshalb ratsam, von jedem H-Antigen, z B H a, mindestens 2 Seren, die zu 2 verschiedenen O-Gruppen gehoren, zu besitzen So kann man z B 2 H a Seren, ein Serum von *S paratyphi-A* (1,2,12 a –) und ein Serum von *S bilthoven* (47 a –) haben Man kann dann sowohl fur die Objektglas-Agglutination als auch fur die Schwärmhemmung oder Isolierung der anderen Phase ein H a-Serum, das keine O-Antikorper fur den betreffenden Stamm enthält, benutzen Auf diese Weise sind Absorptionen überflüssig, sodass man mit den gewohnlichen, nicht absorbierten H Seren arbeiten kann Handelt es sich also um eine Kultur mit den O-Antigenen 47, so benutzt man das H a-Serum von *S paratyphi-A* (1,2,12 a –), während man umgekehrt beim Vorliegen eines *S paratyphi-A*-Stammes das H a Serum von *S bilthoven* anwendet

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[illegible] also nicht alle bekannten, monophasischen Kulturen enthalten, sondern nur solche, die zur Herstellung monophasischer H-Seren von praktischer Bedeutung sind.

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Es wird daher empfohlen, bei der Diagnose von *Salmonella species* für jedes H-Antigen mindestens 2 verschiedene H-Seren, die verschiedene O-Antikörper enthalten, zu verwenden. Die hierzu nötigen Kulturen sind in der Tabelle angegeben, doch kann nicht dafür garantiert werden, dass alle diese Kulturen konstant monophasisch bleiben. Es ist daher notwendig, die betreffenden Stämme vor Gebrauch zu kontrollieren.

Die meisten der in der Tabelle angeführten Kulturen sind in der Natur gefundene, monophasische Stämme. Nur die mit „*Salmonella* (transd.)" bezeichneten Kulturen sind im Laboratorium durch Transduction hergestellte Hybriden und von *P. R. Edwards* oder *J. Lederberg* übersandt. Nähere Angaben über diese transducierten Kulturen sind in folgender Liste enthalten.

Liste der transducierten Kulturen

532 = 9,12 a – = S.W. 1040 = *S. typhi* × *S. sendai*
438 = 9,12 b – = S.W. 670 = *S. typhi* × *S. abony*
531 = 9,12 b – = S.W. 1039 = *S. typhi* × *S. paratyphi-B*
530 = 9,12 c – = S.W. 902 = *S. typhi* × *S. altendorf*
525 = 4,5,12 d – = S.W. 987 = *S. paratyphi-B* × *S. zega*
528 = 9,12 e,h – = S.W. 668 = *S. typhi* × *S. san diego*
437 = 4,5,12 g,p – = S.W. 662 = *S. paratyphi B* × *S. dublin*
436 = 9,12 i – = S.W. 520 = *S. typhi* × *S. typhi murium*
527 = 9,12 i = S.W. 570 = *S. typhi* × *S. typhi-murium*
524 = 4,5,12 l,z_{28} – = S.W. 984 = *S. paratyphi-B* × *S. javiana*
523 = 4,5,12 r = S.W. 683 = *S. paratyphi-B* × *S. heidelberg*
529 = 9,12 r – = S.W. 687 = *S. typhi* × *S. heidelberg*
526 = 4,5,12 – z_6 = S.W. 999 = *S. paratyphi B* × *S. zega*

TABELLE
Monophasische Salmonella Kulturen

Nr	Species	O	H 1 Phase	H 2 Phase
1	S. paratyphi A	1,2,12	a	—
2	S. paratyphi A var. durazzo	2,12	a	—
1307	Salmonella	6,7	a	—
532	Salmonella (transd.)	9,12	a	—
1122	Salmonella	16	a	—
1433	S. assen	21	a	—
570	S. umhlatazana	35	a	—
1321	S. bilthoven	47	a	—
5	S. java	4,5,12	b	—
950	S. sofia	4,12	b	—
31	S. schleissheim	4,12,27	b	—
1101	Salmonella	6,7	b	—
438	Salmonella (transd.)	9,12	b	—

Nr	Species	O	H	
			1 Phase	2 Phase
531	Salmonella (transd)	9 12	b	—
899	Salmonella	11	b	—
252	S atlanta	13 23	b	—
1192	S tucson	1 6 14 25	b	—
218	S minnesota	21	b	—
821	S minnesota	21	b	—
1150	S tchad	35	b	—
453	Salmonella	40	b	—
1395	S vietnam	41	b	—
1128	S saka	47	b	—
1269	S flottbek	52	b	—
1370	S artis	56	b	—
35	S cholerae suis	—	c	—
211	S cholerae suis	6 7	c	—
530	Salmonella (transd)	9 12	c	—
1675	Salmonella	3 10	c	—
711	S yolo	35	c	—
595	Salmonella (transd)	4 5 12	d	—
1[illegible]5	S virginia	(8)	d	—
1126	S virginia	(8)	d	—
57	S typhi (H 901)	9 12	d	—
835	S wichita	13 23	d	—
10	S wichita	1 13 23	d	—
1461	S carletonville	38	d	—
1395	S egusi	41	d	—
1574	S gokul	1 51	d	—
146	Salmonella	4 5 12	e h	—
360	Salmonella	4 5 12	e h	—
528	Salmonella (transd)	9 12	e h	—
730	S anatum	3 10	e h	—
731	S anatum	3 10	e h	—
1163	S mara	39	e h	—
1017	S kiel	1 2 12	g p	—
437	Salmonella (transd)	4 5 12	g p	—
209	S typhi murium	4 5 12	i	—
1645	Salmonella	(8) 20	i	—
436	Salmonella (transd)	9 12	i	—
577	Salmonella (transd)	9 12	i	—
1419	Salmonella	13 23	i	—
212	S thompson	6 7	k	—
213	S thompson	6 7	k	—
910	S bredeney	1 4 12 27	l v	—
5[illegible]4	Salmonella (transd)	4 5 12	l z [illegible]	—
14	S javiana	1 9 12	l z [illegible]	—
818	Salmonella	3 10	l v	—
819	Salmonella	3 10	l v	—
911	S westerstede	1 3 19	l z_{13}	—
216	S worthington	1 13 23	l w	—
363	S worthington	1 13 23	l w	—
642	Salmonella	16	l v	—
1091	Salmonella	16	l v	—

Es wird daher empfohlen, bei der Diagnose von *Salmonella species* fur jedes H-Antigen mindestens 2 verschiedene H-Seren, die verschiedene O-Antikorper enthalten, zu verwenden Die hierzu notigen Kulturen sind in der Tabelle angegeben, doch kann nicht dafur garantiert werden, dass alle diese Kulturen konstant monophasisch bleiben Es ist daher notwendig, die betreffenden Stamme vor Gebrauch zu kontrollieren

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438 = 9,12 b – = S W 670 = *S typhi* × *S abony*
531 = 9,12 b – = S W 1039 = *S typhi* × *S paratyphi-B*
530 = 9,12 c = S W 902 = *S typhi* × *S altendorf*
525 = 4,5,12 d – = S W 987 = *S paratyphi-B* × *S zega*
528 = 9,12 e,h – = S W 668 = *S typhi* × *S san-diego*
437 = 4,5,12 g,p = S W 662 = *S paratyphi B* × *S dublin*
436 = 9,12 i – = S W 520 = *S typhi* × *S typhi-murium*
527 = 9 12 i = S W 570 = *S typhi* × *S typhi-murium*
524 = 4,5,12 l,z 28 = S W 984 = *S paratyphi B* × *S javiana*
523 = 4,5,12 r = S W 683 = *S paratyphi B* × *S heidelberg*
529 = 9,12 r – = S W 687 = *S typhi* × *S heidelberg*
526 = 4,5,12 – ze = S W 999 = *S paratyphi-B* × *S zega*

TABELLE
Monophasische Salmonella Kulturen

Nr	Species	O	H	
			1 Phase	2 Phase
1	S paratyphi A	1 2 12	a	—
2	S paratyphi A var durazzo	2 12	a	—
1307	Salmonella	6 7	a	—
532	Salmonella (transd)	9 12	a	—
1122	Salmonella	16	a	—
1433	S assen	21	a	—
570	S umhlatazana	35	a	—
1321	S bilthoven	47	a	—
5	S java	4 5 12	b	—
950	S sofia	4 12	b	—
31	S schleissheim	4 12 27	b	—
1101	Salmonella	6 7	b	—
438	Salmonella (transd)	9 12	b	—

Nr	Species	O	H	
			1 Phase	2 Phase
592	Salmonella	4 5 12	—	1 2
1185	Salmonella	4 12	—	1 6
596	Salmonella (transd.)	4 5 12	—	z6
36	S. cholerae suis	6 7	—	1 5
37	S. cholerae suis	6 7	—	1 5
908	Salmonella	6 7	—	1 6
51	S. newport var. puerto rico	6 8	—	1 2
215	S. javiana	1 9 12	—	1 5
1538	Salmonella	9 12	—	1 6
879	Salmonella	3 10	—	1 6
1311	Salmonella	40	—	1 5 7
1331	S. lobatsi	52	—	1 5 7

ZUSAMMENFASSUNG

Es werden monophasische *Salmonella*-Kulturen, die zur Herstellung von H-Seren benutzt werden können, in einer Tabelle zusammengestellt. Es wird empfohlen, bei der Diagnose von *Salmonella species* für jedes H-Antigen mindestens 2 verschiedene H-Seren, die verschiedene O-Antikörper enthalten, zu verwenden.

LITERATUR

Edwards, P. R. & Bruner, D. W.: "Notes on monophasic Salmonella cultures and their use in the production of diagnostic serums." J. Bacter. 52, 493-498, 1946.

Kauffmann, F.: Die Bakteriologie der Salmonella-Species. Munksgaard, Kopenhagen 1961.

Nachtrag bei der Korrektur

Alle in dieser Arbeit erwähnten Kulturen sind bei Dr. *R. Rohde*, Salmonella-Centrale, Hygiene-Institut, Gorch-Fock-Wall 15/17, Hamburg 36, deponiert und können bei ihm bestellt werden.

Nr	Species	O	H	
			1 Phase	2 Phase
523	Salmonella (transd.)	4 5 12	r	—
529	Salmonella (transd.)	9 12	r	—
353	S nairobi	42	r	—
1053	S nairobi	42	r	—
232	Salmonella	6 7	y	—
799	Salmonella	3 10	y	—
217	S madelia	1 6 14 25	y	—
305	Salmonella	28	y	—
782	Salmonella	28	y	—
219	Salmonella	28	y	—
633	Salmonella	13 23	z	—
367	S illinois	(3), (15) 34	z_{10}	—
688	S guinea	44	z_{10}	—
1072	S ngozi	48	z_{10}	—
207	S brancaster	1 4 12	z_{29}	—
142	S tennessee	6 7	z_{29}	—
949	S uno	6 8	z_{29}	—
885	S tamale	(8) 20	z_{29}	—
1039	S ouakam	(9) 46	z_{29}	—
920	S jedburgh	3 10	z_{29}	—
194	S cubana	1 13 23	z_{29}	—
552	S jacksonville	16	z_{29}	—
1023	S kandla	17	z_{29}	—
1611	S djermaia	28	z_{29}	—
860	S omifisan	40	z_{29}	—
1061	S jodhpur	45	z_{29}	—
1635	Salmonella	4 12	z_3	—
1404	S tejas	4 12	z_{36}	—
1672	Salmonella	6 7	z_{36}	—
273	S macallen	3 10	z	—
1423	S beloha	18	z_{36}	—
247	S weslaco	42	z_3	—
1308	S wilhelmsburg	4 12 27	z_{38}	—
504	S lille	6 7	z_{38}	—
1189	S bornum	6 (7) (14)	z_{38}	—
510	S fresno	(9) 46	z_{38}	—
1080	S bolombo	3 10	z_{38}	—
1582	S leiden	13 22	z_{38}	—
684	S fanti	13 23	z_{38}	—
1673	S sara	1 6 14 25	z_{38}	—
1612	S synthia	18	z_{38}	—
1172	S aderike	28	z_{38}	—
918	S offa	41	z_{38}	—
1475	S irigny	43	z_{38}	—
26	S abortus equi	4 12	—	e n x
270	S abortus equi	4 12	—	e n x
907	Pc 230	4 12	—	e n z_{15}
739	Citrobacter	13 22	—	e n
281	S paratyphi A	1 2 12	—	1 5
368	S paratyphi A	1 2 12	—	1 5

beaker as a mixing chamber which by a syphon was connected with a 1 000 ml Erlenmeyer flask containing 900 ml of starting buffer supplemented with 0 5 M NaCl The flow rate was adjusted to 150 ml hour 12 ml fractions of effluent being collected The β_{1C} globulin effluent zone was localized with the aid of immuno electrophoresis or Ouchterlony double diffusion technique plates and the antiserum

0 tubes
buffer
ice with
H 7.2 μ

0 1 containing 0 9 per cent NaCl The temperature was kept at +4° C during the entire isolation procedure This simplified isolation procedure gave a yield of about 100 mg of protein of which β_{1C} globulin represented about 75 per cent Contamination with 5–10 per cent β_{1A} globulin could usually not be avoided

β_{1C} globulin was isolated also on a few occasions according to *Muller Eberhard et al* (1960) This method gave a purer preparation but the method is much more timeconsuming No differences between reactions of the preparations were seen

The β_{1C} globulin was stored at +4° C for at most a week and before use it was

... 12 hours at +4° C the precipitate was spun down resuspended and washed twice with physiological saline The immunoaggregates were then used immediately

Agar gel electrophoresis was run according to *Lundh* (1964) Four samples were run on each glass plate In order to vary the endosmotic flow all preparations were separated on agar gel prepared from three different types of agar ...

... in the same way as the before mentioned agar gel electrophoresis A specially constructed twinbladed knife was used for cutting out two 20 μl slits in the agar gel With the aid of this knife a well was also cut in the middle of the gel Uniform position of the slits was obtained ...
template ...
well had ...
stand at ...

... out according to *Lundh*

Quantitative determination of total protein was carried out with the biuret method (*Lundh* 1963)

EXPERIMENTAL

A The Effect of Incubation with Hydrazine in Different Concentrations and at Different Temperatures

Samples with serum as well as serum to which β_{1C} globulin had been added and isolated β_{1C} globulin solution were treated according to

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THE EFFECT OF INCUBATION WITH HYDRAZINE AND IMMUNOPRECIPITATE ON β_{1C}-GLOBULIN

By

B. LUNDH

Received 31.viii.64

Soon after the isolation of β_{1C}-globulin (*Müller-Eberhard, Nilsson & Aronsson* 1960) reports appeared on the lability of this protein, its conversion to β_{1A}-globulin and its relation to the serum complement (*Müller-Eberhard & Nilsson* 1960, *Müller-Eberhard* 1961). Independently of *Müller-Eberhard & Nilsson*, *Zimmer & Woringer* (1959) showed that the immunoelectrophoretic β_1 region included a labile protein, which on storage of the serum was gradually converted into a component of higher mobility.

Zimmer (1960, 1961) also showed that the conversion could be accelerated by means of "traitement ammoniacal" or by incubation with immunoprecipitate. The final products appeared to be the same whether one or the other method was used. The conversion of β_{1C}-globulin to β_{1A}-globulin was studied also by *Peetom & Pondman* (1961), who concluded that treatment with hydrazine and incubation with immunoprecipitate yield identical final products. The experiments forming the basis of the present investigation have shown, however, that the results of incubation of β_{1C}-globulin with hydrazine, respectively immunoprecipitate, are not identical.

MATERIAL AND METHODS

Fresh native serum. Blood collected from apparently healthy persons was allowed to stand one hour at room temperature, after which the serum was separated by centrifugation. The electrophoretic analysis was started immediately.

β_{1C}-globulin. β_{1C}-globulin was isolated by means of ion exchange chromatography (modif. *Harboe, Müller-Eberhard, Fudenberg, Polley & Mollison* 1963) of patients' serum stored at room temperature for 8 hours and then pooled. A column with DEAE cellulose, 50 × 3.5 cm, was equilibrated with a phosphate buffer, pH 7.2, μ 0.02 (starting buffer). After dialysis for 15 hours against starting buffer, 100 ml of serum was applied to the column. The chromatogram was developed with a salt gradient. This gradient was produced with 2 000 ml of starting buffer in a 2 000 ml

This investigation has been supported by grants from *Public Health Service Research Grant F 5703-2 from the National Institutes of Health*, from the *Medical Faculty, University of Lund* and from *Alfred Österlunds stiftelse*.

For technical assistance I thank Mrs. *Karin Pettersson*.

beaker as a mixing chamber which by a syphon was connected with a 1 000 ml Erlenmeyer flask containing 900 ml of starting buffer supplemented with 0 5 M NaCl The flow rate was adjusted to 150 ml hour 12 ml fractions of effluent being collected The β_{1C} globulin effluent zone was localized with the aid of immuno electrophoresis or Ouchterlony double diffusion technique plates and the antiserum mentioned below As a rule β_{1C} globulin could be demonstrated in about 30 tubes The middle ones were pooled and dialysed for 24 hours against a phosphate buffer pH 5 4 μ 0 02 The precipitate was spun down resuspended and washed twice with ice cold buffer The precipitate was then dissolved in phosphate buffer pH 7 2 μ 0 1 containing 0 9 per cent NaCl The temperature was kept at +4° C during the entire isolation procedure This simplified isolation procedure gave a yield of about 100 mg of protein of which β_{1C} globulin represented about 75 per cent Contamination with 5 10 per cent β_{1A} globulin could usually not be avoided

β_{1C} globulin was isolated also on a few occasions according to *Muller Eberhard et al* (1960) This method gave a purer preparation but the method is much more timeconsuming No differences between reactions of the preparations were seen

The β_{1C} globulin was stored at +4° C for at most a week and before use it was dialysed for one hour during agitation in the cold against the same barbital buffer

a ...

...

... and 12 hours at +4° C the precipitate was spun down resuspended and washed twice with physiological saline The immunoaggregates were then used immediately

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... the aid of this knife a well was also cut in the middle of the gel Uniform position of the slits was obtained by the use of template ...

... saline

Quantitative determination of β_{1C} globulin was carried out according to *Lundh* 1964

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...

ad

TABLE 1

Tube	Sample ml	Hydrazine		NaCl 0.9% ml	Temp	Time min
		0.1 M ml	1.0 M ml			
1	1	–	–	0.05	+0°	30
2	1	0.05	–	–	+37°	30
3	1	–	0.05	–	+37°	30
4	1	–	–	0.05	+37°	30
5	1	0.05	–	–	+50°	30
6	1	–	0.05	–	+50°	30
7	1	–	–	0.05	+50°	30

Table 1 All the test tubes were then placed in an ice-bath before electrophoresis in agar gel, which was started without delay

RESULTS AND COMMENTS

Fig 1 gives the results obtained with some of the preparations in the different types of agar gel and Fig 2 shows examples of the results of the immunoelectrophoretic analysis of the serum to which β_{1C}-globulin had been added It is clear from the illustrations that treatment with hydrazine at +37° C results in diminution and final disappearance of the β_{1C}-globulin and the simultaneous appearance of two new components of different electrophoretic mobility In agar gel prepared with Special Agar-Noble, one of the new fractions migrated between the *original β_{1C}-band and the application slit*, while the other was accumulated at the anodal end of the application slit

In agarose gel, where, owing to the slower endosmotic flow the β_{1C}-globulin is located anodally to the application slit, one of the new fractions is seen cathodally and the other anodally to the original site of the β_{1C}-globulin That these two fractions of different electrophoretic mobility have the same antigen determinants as the original β_{1C}-globulin was confirmed by immunoelectrophoretic analysis In Reinagar only one new component was seen and here *at the application slit* This might be explained by the assumption that the migration of one of the components was compensated by the endosmotic flow with the result that the component remained at the site of application and that the properties of the other were such that it could not migrate in the gel After treatment with hydrazine at +50° C electrophoresis in Special Agar Noble and Reinagar resulted in a total extinction of the β_{1C}-band and an increased amount of stained substance in the anodal end of the application slit, while in agarose gel only a diffusely outlined *band* was seen, and then just anodally of the original site of the β_{1C}-globulin band

B *Incubation with Immunoprecipitate*

Immunoprecipitate was added in increasing amounts to a standardized amount of normal serum The precipitate was suspended carefully and

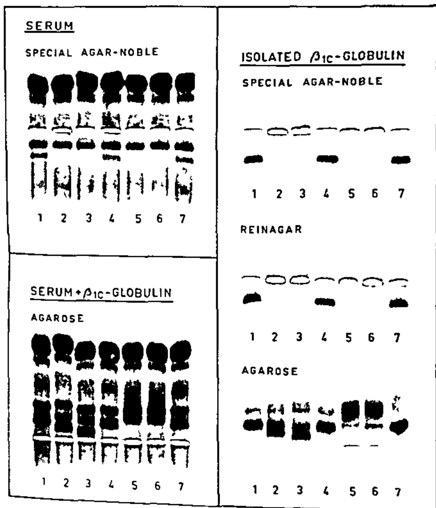


Fig 1

[illegible] refer to Table I

then incubated at + 37° C in a rocking bed for 60 minutes The samples were then centrifuged at 3,000 × g for 10 minutes and the supernatants were preserved for determination of the amount of β_{1}c-globulin consumed and of the total protein as well as for conventional agar gel electrophoresis and immunoelectrophoresis Similar experiments were carried out also with different dilutions of patients' pooled serum containing an increased amount of the β_{1}c-globulin

Fig 3 shows the results of conventional and immunoelectrophoretic

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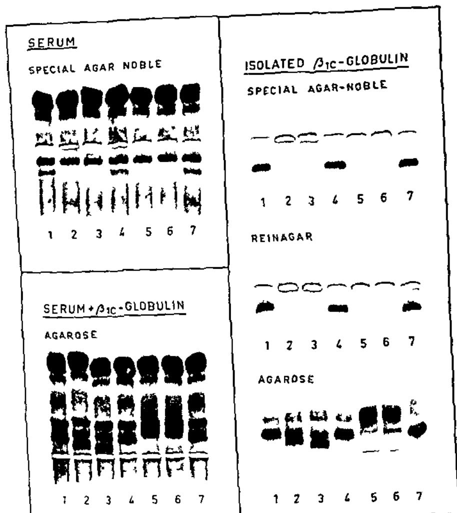


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u c a s Icler t Table I

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3	1	–	0.05	–	+37°	30
4	1	–	–	0.05	+37°	30
5	1	0.05	–	–	+50°	30
6	1	–	0.05	–	+50°	30
7	1	–	–	0.05	+50°	30

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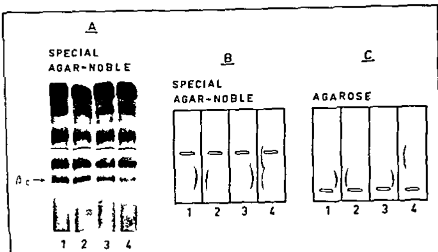


Fig 3

The result f incubation of serum with immunoprecipitate in increasing amount

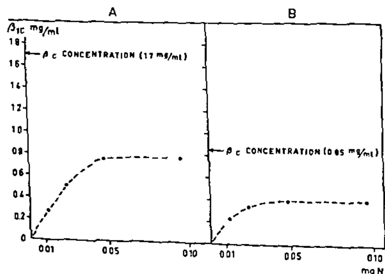


Fig 4

The am unt f β_{1C} gl bulin consumed during incul ati n f native serum at +37° C for 60 min with immunoprec p tate in increasing am unt
A Pat ents pooled serum B The same serum dil ute l 1 + 1

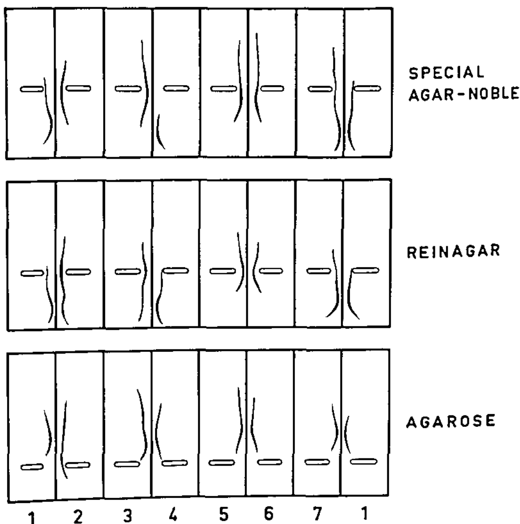


Fig 2

Schematic drawings of the results of the immunoelectrophoretic analyses on different sorts of agar gel of serum to which β_{1C} globulin had been added after incubation with different amounts of hydrazine and at different temperatures Numerals refer to Table 1

fractionation of fresh serum incubated with immunoprecipitate in increasing concentration

Fig 4 gives the amount of β_{1C} globulin consumed after incubation with immunoprecipitate in increasing amount of the patients' pooled serum containing an increased amount of β_{1C}-globulin and of this pool diluted 1 + 1 The results were not corrected for total protein because the dilution on addition of immunoprecipitate was regularly less than five per cent

On incubation with immunoprecipitate part of the β_{1C}-globulin is converted into a component, which in all types of agar gel migrated

It should be mentioned that hydrazine has an effect also on isolated β_C globulin irrespective of the method used for isolation of the latter This is not in accord with *Fjellstrom* s (1963) observation that hydrazine has no effect on isolated β_C globulin demonstrable with starch gel electrophoresis This discrepancy can probably be explained by the assumption that Fjellstrom used a relatively small amount of hydrazine in relation to the amount of β_C globulin and that the small changes that occurred escaped attention When a higher concentration of hydrazine is used or when the sensitivity of the recording is increased *e g* by supplementing starch gel electrophoresis with immunodiffusion the effect of hydrazine on isolated β_{1C} globulin can also be demonstrated in starch gel electrophoresis (*Lundh* to be published)

Incubation with immunoprecipitate converts β_{1C} globulin into β_{1A} globulin only which has also been described by previous authors (*Muller Eberhard & Nilsson* 1960 *Zimmer* 1961 *Peetom & Pondman* 1961) In agar gel electrophoresis the mobility of this component is the same as that of the protein that forms on storage for a long time of β_C globulin The simultaneous measurements of the β_C globulin consumption must be regarded as very approximate because the changes in several of the samples were small and the method used is not very exact (*Lundh* 1964) It must be realized however that under the present conditions of the investigation the concentration of β_C globulin is not the limiting factor for conversion of β_{1C} globulin to β_{1A} globulin

Therefore there was no reason to carry the quantitative determinations further after corresponding experiments with serum enriched with β_C globulin Qualitative analysis by immunoelectrophoresis showed the same changes in serum to which β_C globulin had been added as in native serum and with isolated β_C globulin solution no change could be observed after incubation with immunoprecipitate

SUMMARY

Serum to which isolated β_C globulin had been added and isolated β_C globulin were incubated with hydrazine in different concentrations and at different temperatures and with immunoprecipitate The samples were then analysed by electrophoresis and immunoelectrophoresis on agar gels with varying endosmotic flow After treatment with hydrazine not only β_A globulin was observed but also a large component probably consisting of a larger molecule On incubation with immunoprecipitate only β_A globulin was formed

Incubation with immunoprecipitate in increasing concentration

more rapidly than the β_{1C} globulin In the system used this component migrated at the same rate as the protein obtained on storage of β_{1C} globulin at +4° C *for more than two months The figures also show* that even large amounts of immunoprecipitate will not convert all the β_{1C}-globulin in a serum into this component Incubation of isolated β_{1C}-globulin with immunoprecipitate was not found to affect the β_{1C}-globulin

DISCUSSION

According to previous investigations on the lability of β_{1C}-globulin this protein changes during storage as well as after decomplementation with immunoprecipitate and after incubation with hydrazine into a single new component called β_{1A}-globulin (*Muller-Eberhard & Nilsson* 1960, *Muller-Eberhard* 1961, *Peetom & Pondman* 1961, *Steinbuch* 1964)

Observations made in the present investigation, however, suggest that the final products vary After treatment of the different samples with hydrazine two new components appeared with different electrophoretic mobilities One of them probably corresponds to the original β_{1A} globulin described by *Muller Eberhard et al* (1960) The other and larger component appears to be modified by hydrazine in such a way that it cannot migrate in agar gel, or migrates only slightly Such modification may consist of a change in molecular weight, such as a polymerization, a change in form or in charge with consequent inhibition of mobility This latter and larger component has not been described before and has been provisionally called β_{1AH} (β_{1A} Hydrazine)

Incubation with heat alone (at +37° C or + 50° C for 30 min) results in conversion of a very small part of β_{1C}-globulin to β_{1A}-globulin while on hydrazine treatment the bulk of the β_{1C} globulin is converted into the β_{1AH}-*component as a direct effect of the hydrazine, and a minor part* to β_{1A}-globulin as an effect of heating The reason why the β_{1AH}-component has hitherto remained concealed is probably that previous investigators used microimmunoelectrophoresis, which does not give such a good power of resolution or, it may be attributable to the use of agar (Special Agar-Noble, Reinagar) in which the β_{1AH}-component cannot migrate The β_{1A}-globulin found in the sample incubated with hydrazine was then interpreted as a direct consequence of the treatment with hydrazine, especially as no other new components were observed

The results obtained after incubation with hydrazine at *+50° C are* more difficult to interpret On electrophoresis in Special Agar-Noble and Reinagar no essential difference could be seen, while electrophoresis in agarose gel produced quite a different pattern β_{1AH}, which showed up very distinctly after incubation at +37° C was now missing and instead a diffuse band was seen with roughly the same mobility as the β_{1A} globulin But it was evidently not β_{1A} globulin since no β_{1A}-globulin was seen in the gel prepared with Special Agar-Noble or Rein agar

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β_{1C} GLOBULIN ON STARCH GEL ELECTROPHORESIS

By

B LUNDH

Received 31 viii 64

The largest component of the serum complement system is the β_{1C}-globulin, whose concentration is normally about 100 mg/100 ml serum (*Lundh* 1964) This is more than enough to produce a distinct band in the pattern obtained on starch gel electrophoresis *Fjellström* (1963a) studied the complement components of the serum by means of starch gel electrophoresis according to *Poulik*'s (1957) modification He showed that isolated β_{1C} globulin differed in mobility from the two bands situated immediately cathodally to the transferrin band and which Fjellström called X and X_1 in order of distance from the anode X_1 was believed to represent a complement component (C'_2) In their elaborations of methods for isolating β_{1A} and β_{1C}-globulin, respectively, *Schultze Heide & Haupt* (1962) and *Steinbuch* (1963) gave reports on the mobilities of these proteins on starch gel electrophoresis

In the present investigation, however, it was found that the serum β_{1C}-globulin unlike most other proteins, changes in association with conventional electrophoresis on starch gel The β_{1C} globulin modified by starch gel electrophoresis is compared with the final products formed after storage and on incubation of this plasma protein with hydrazine or immunoprecipitate

METHODS

Starch gel electrophoresis was run according to the original method of *Smithies* (1955) as well as according the the method of *Poulik* (1957) with a discontinous buffer system Unless otherwise stated the separations were run at room temperature In some of the experiments the buffers were supplemented with EDTA to a final concentration of 0 0005 M (*Fjellström* 1963b)

All the separations were [illegible]

[illegible] has been supported by grants from *Public Health Service Research Grant E-4703 2* from the *National Institutes of Health* from the *Medical Faculty University of Lund* and from "*Alfred Österlunds stiftelse*

For technical assistance I thank Mrs *Karin Pettersson*

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Acta path et microbiol scandinav 63 275–280 1965

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β_{1C}-GLOBULIN ON STARCH GEL ELECTROPHORESIS

By

B Lundh

Received 31 viii 64

The largest component of the serum complement system is the β_{1C}-globulin, whose concentration is normally about 100 mg/100 ml serum (*Lundh* 1964) This is more than enough to produce a distinct band in the pattern obtained on starch gel electrophoresis *Fjellström* (1963a) studied the complement components of the serum by means of starch gel electrophoresis according to *Poulik*'s (1957) modification He showed that isolated β_{1C}-globulin differed in mobility from the two bands situated immediately cathodally to the transferrin band and which Fjellström called X_2 and X_1 in order of distance from the anode. X_1 was believed to represent a complement component (C'_2). In their elaborations of methods for isolating β_{1A}-and β_{1C}-globulin, respectively, *Schultze, Heide & Haupt* (1962) and *Steinbuch* (1963) gave reports on the mobilities of these proteins on starch gel electrophoresis

In the present investigation, however, it was found that the serum β_{1C}-globulin, unlike most other proteins, changes in association with conventional electrophoresis on starch gel The β_{1C}-globulin modified by starch gel electrophoresis is compared with the final products formed after storage and on incubation of this plasma protein with hydrazine or immunoprecipitate

METHODS

Starch [illegible]
(1955) [illegible]
buffer [illegible]
In some [illegible]
concent[illegible] (*Fjellström* 1963b)
All the separations [illegible]
following way After [illegible]
halves was stained [illegible]

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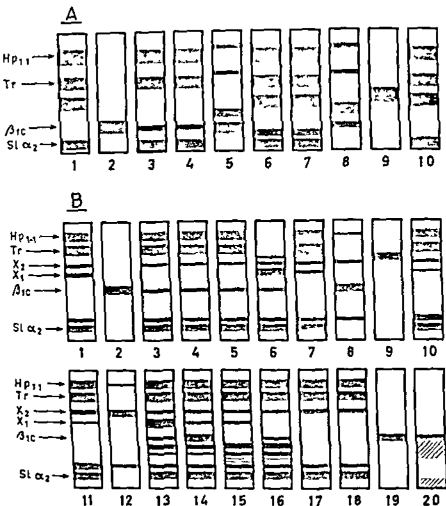


Fig 1

Schematic drawings of the result of separation on starch gel Unless otherwise stated the serum belongs to Hp group 1 1 A Separation according to the method of Smithies of 1 Native serum 2 Isolated β_{1C} globulin 3 Heat inactivated native serum …

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middle of the agar gel a well 300 × 1 5 mm was cut Three starch gel segments were then placed on the agar gel 8 mm from the slit three on each side The well was then filled with antiserum which contained antibodies principally against β lipoproteins and β_{1C} β_{1A} *globulins The precipitin lines* were allowed 48 hours to develop The gels were then washed dried and stained with Amidoblack

Treatment with hydrazine and quantitative estimation of β_{1C} globulin was carried out according to *Lundh* (1964)

MATERIAL

The following preparations were analysed

a) Fresh sera from subjectively healthy persons with different haptoglobin groups

b) The same sera heated to +50° C for 30 minutes

c) The same sera with addition of 20 µl 10 per cent dextran sulphate (Sulphate de Dextrane, l'Equilibre Biologique S A Commentry (Allier) France) /ml serum resp 20 µl 5 per cent Heparin (Vitrum Stockholm Sweden 5 000 U/ml) /ml serum

d) The same sera stored for 7 days at +4° C

e) The same sera treated with hydrazine

f) The same sera incubated with immunoprecipitate (for details see *Lundh* 1965)

g) A euglobulin preparation This was prepared by dialysis of 250 ml of pooled fresh serum against 10 l of phosphate buffer pH 5 4 ionic strength 0 02 for 20 hours at +4° C The precipitate was spun down resuspended and washed twice in ice cold buffer and then dissolved in 10 ml of phosphate buffer, pH 7 0 ionic strength 0 1 to which was added NaCl in a final concentration of 0 145 M

h) β_{1C} globulin which was isolated according to *Muller Eberhard Nilsson & Aronsson* (1960)

i) β_{1A} globulin which was obtained by storing β_{1C} globulin for more than two months at +4° C.

RESULTS

Fig 1A and 1B show some of the patterns obtained on starch gel electrophoresis according to Smithies, respectively to Poulik It is clear from the illustrations that native serum from an individual with haptoglobin 1 1 showed no component with the same mobility as isolated β_{1C} globulin Not until after heating or addition of heparin or dextran sulphate was any fraction seen at the expected site Addition of EDTA to the gel and to the electrode vessels gave β_{1C} globulin its "natural" mobility On the other hand, the pattern was the same whether separation was done at +4° C or at room temperature

No difference was found between the mobility of β_{1C} globulin in a euglobulin preparation, in which it was the largest component, and that of isolated β_{1C}-globulin

The appearance of this fraction with the same mobility as isolated β_{1C}-globulin was accompanied by the disappearance of the more cathodal (X_1) of the two bands (X and X_1) seen cathodally to the transferrin band on electrophoresis according to Poulik

On electrophoresis of native serum in different types of agar gel, of serum heated to +50° C for 30 minutes, respectively of serum with an addition of heparin or dextran sulphate, the mobility of β_{1C} globulin was always the same

Supplementary examination with immunodiffusion analysis and the use of specific antiserum showed that the band that appeared after heat

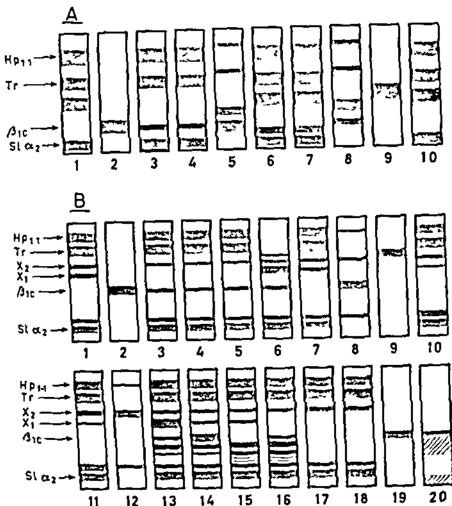


Fig 1

Schematic drawings of the result of separation on starch gel Unless otherwise stated the serum belongs to Hp group 1 1 A Separation according to the method of Smithies of 1 Native serum 2 Isolated β_{1C} globulin 3 Heat inactivated native serum 4 Serum to which dextran sulphate has been added 5 A euglobulin preparati n 6 Nati [illegible]
with hydrazine
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serum 4 Serum [illegible] which dextran sulphate has been added 5 Ser[illegible]
[illegible] inactivated serum
[illegible] group 2 2 17 Aged serum 18 Serum incubated with immunoprecipitate 19 Isolated β_{1C} gl bulin 20 Isolated β_{1C} globulin treated with hydrazine

inactivation etc, had antigen determinants of the same type as the band (X_1) that disappeared simultaneously from the electropherogram

Treatment of native serum with hydrazine resulted in a weakening of X_1, and corresponding treatment of serum *that had been heated, or to* which dextran sulphate or heparin had been added, resulted in a disappearance of the band with the mobility of isolated β_{1C} globulin, and simultaneous broadening of X_2. The same pattern was seen whether the serum, native or inactivated by heat or containing heparin, was allowed to *stand at* $+4°$ C *for 7 days (Fig 1B, 10–12, 17)*

Pure β_{1A}-globulin by itself as well as when added to serum, migrated, however, between transferrin and X_2. Incubation of serum with immunoprecipitate in excess resulted in extinction of X_1 and an increase in breadth of X_2 (Fig 1B, 9, 18)

On exposure of isolated β_{1C}*-globulin* to hydrazine in a *final concentration* of 0 005 M the β_{1C}-band diminished slightly, and when the hydrazine concentration was increased to 0 05 M the band diminished more substantially At the same time two diffuse bands appeared between the β_{1C}-band and the site of application (Fig 1B 19, 20)

DISCUSSION

It is clear from the control experiments with agar gel electrophoresis that the mobility of β_{1C}-globulin was largely the same whether native serum was used or serum that had been heated at $+50°$ C for 30 minutes or serum to which heparin had been added But when these samples were run on starch gel electrophoresis, one of the bands was missing and a new band appeared with a mobility corresponding to that of native, isolated β_{1C}-globulin With the aid of specific antiserum it was found that the two bands consisted of proteins with antigen determinants common to both This suggests that starch gel electrophoresis interferes with the β_{1C}-globulin with consequent modification of the properties and thereby of the electrophoretic mobility of the globulin This interference is not direct but mediated by some serum factor that can be inhibited *by heating at* $+50°$ *C for 30* minutes, or *by addition* of heparin or dextran sulphate Neither is the factor enriched in the euglobulins, since β_{1C}-globulin in a euglobulin preparation migrated at its ordinary rate *Fjellstrom* (1963b) discussed the possibility of interference of complement proteins by starch gel, but made no attempts to check the idea *He stated that of the two bands observed immediately* cathodally to the transferrin, X_1 represented a complement component (C'_3) But it is clear from the findings set forth in preceding paragraphs that the band consists of β_{1C} globulin modified by starch gel electrophoresis This may help to explain *Fjellstrom*'s (1963c) observation that the X_1 band is *more* marked in cardiac infarction, the β_{1C} concentration being increased in this condition, as in other active processes such as pneumonia (*Lundh*, to be published)

The β_{1C} globulin modified in association with starch gel electrophoresis has not the same mobility as the products obtained after storage or after incubation of the serum with immunoprecipitate The mobility of these fractions, on the other hand, is not the same as that of β_{1A} globulin obtained by storage of β_{1C}-globulin for more than two months at +4° C, for, as shown in Fig 1B and reported earlier by *Fjellström* (1963), for example, on starch gel electrophoresis according to Poulik's technique β_{1A} migrates between X_2 and the transferrin

As to hydrazine and β_{1C}-globulin, *Fjellstrom* (1963a) reported that no effect was obtained on isolated protein This assertion could not be confirmed by observations made in the present investigation, in which *addition of hydrazine in increasing concentration* to the sample was followed by a successive decrease and disappearance of the β_{1C}-band, and simultaneous appearance of two diffuse zones between the β_{1C}-globulin and the site of application This is in agreement with findings made on agar gel electrophoresis (*Lundh* 1965), namely that hydrazine affects also isolated β_{1C} globulin and that the final products migrate but little or not at all in agar gel This lack of migration in agar gel was found to be the same also for serum and for serum to which isolated β_{1C} globulin had been added But this was not the case on starch gel electrophoresis On incubation, with hydrazine, of native serum, heat inactivated serum or serum to which heparin or dextrane sulphate had been added, a component was obtained with a mobility corresponding to that of X_2 The mobility was thus the same as that of the β_{1A}-globulin obtained on incubation with immunoprecipitate or when the serum was old This means that, analysed on starch gel electrophoresis, the decomposition products of the β_{1C} globulin fall into 4 groups (a) the component demonstrable after storage or after incubation of serum with immunoprecipitate respectively with hydrazine and migrating at the same rate as X_2 (b) the component appearing on storage of isolated β_{1C} globulin (c) the probably large molecular components, demonstrable after treatment of isolated β_{1C}-globulin with hydrazine and (d) the component located in X_1 and occurring on interference of β_{1C} globulin by the starch gel under the influence of some hitherto undefined serum factor

Fjellstrom (1963a) suggested the possibility of X_1 and X_2 containing different types of β_{1A} globulin but this was regarded as less likely, because the mobility was not the same as that of isolated β_{1A} globulin (aged β_{1C} globulin)

As to the interference between starch gel and β_{1C} globulin, it may be stated that the mediating factor is thermolabile and that it is inactivated by heparin dextran sulphate and EDTA, but not by exposure to +4° C Incubation of serum with an equal weight of finely pulverized starch gel was found to have no demonstrable effect on the β_{1C}-globulin analysed on agar gel electrophoresis (*Lundh* unpublished) This negative result may have been due to the use of an insufficient amount of starch

gel for fear of overdilution of serum. The serum may also contain some substance that inhibits the reaction under consideration and that is readily separated off on electrophoresis.

That interference occurs between the starch gel and complement system is not surprising. Starch gel is built up on a core of polysaccharides, which are known to have an inactivating effect on complement, and then particularly on C'_3, of which β_{1C} globulin is a component. The inhibition of the interference is interesting, as are the different ways in which it occurs. The fact that the interference can be inhibited by heating and by EDTA and in a euglobulin preparation and that it is not inhibited at +4° C suggests that C'_2 takes part in the reaction. That heparin should also act via C'_2 is probable but not proven. It should, however, be mentioned that heparin as well as dextran sulphate in the concentrations used completely inhibit the conversion of β_{1C}-globulin to β_{1A} globulin on incubation with immunoprecipitate (*Lundh*, to be *published*).

SUMMARY

The mobility of β_{1C}-globulin and that of its decomposition products on starch gel electrophoresis according to Poulik and to Smithies are demonstrated.

Interference was demonstrated between starch gel and β_{1C} globulin. The occurrence of this interference requires the presence of a thermolabile serum factor, which is inactivated by heparin, for example.

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THE RADIATION RESISTANCE OF SUBSTRAINS FROM *STREPTOCOCCUS FAECIUM* SELECTED AFTER IRRADIATION OF TWO DIFFERENT STRAINS

By

EBBE AHRENSBURG CHRISTENSEN and EBBE KJEMS

Received 11 ix 64

It is well known that the incidence of mutation is increased by exposure to ionizing radiation It has been shown that as a result of irradiation with ultraviolet light it is possible to induce formation of strains of *Escherichia coli* which are more resistant to ionizing radiation than the mother strain (12, 19) Erdman et al showed in 1961 that repeated gamma irradiation could increase the radiation resistance of cultures of both *Escherichia coli* and *Streptococcus faecalis*, as well as of one strain of *Staphylococcus aureus* and vegetative forms of *Clostridium botulinum* while no increase in resistance could be demonstrated in cultures of two other strains of *St aureus* and in spores of *Cl botulinum* (8)

Previously it has been shown that in the dry state, *Streptococcus faecium* possesses considerable resistance to ionizing radiation (2) In the present study a number of substrains were selected from two faecium strains after the latter had been exposed to gamma or electron irradiation with doses between 1 2 and 3 5 Mrad The resistance of the mother strains and the substrains to ionizing radiation was then examined by determining the inactivation curves of the strains

MATERIAL AND METHODS

Th

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At

w [illegible] from irradiated dry test pieces

w [illegible] were designated F strains substrains isolated from irradiated F strains were designated G strains and finally substra [illegible]

stra [illegible]

of the strains were selected at random as representatives of the majority of the colonies which had not altered their appearance with respect to the size and the zone of haemolysis after the irradiation (apart from the delay in colony formation always observed following irradiation with high doses) Such strains are marked with the letter *a* on Figs 1 and 3 Other substrains were selected on account of their deviant colony size or on account of essential changes in the size of the zone of haemolysis on the plates from the primary culturing after irradiation Where such changes were visible only on the primary plates or maintained through only a single or a few subcultures the strains on Figs 1 and 3 are marked *b* Where the change remained stable through more than five subcultures from single colonies the strains are marked c on Figs 1 and 3

Nine of a total of 32 substrains were isolated from the substrains mentioned above without an intervening irradiation Some of the strains isolated from the first culture after the irradiation could in one of the first subcultures be split into for example a rapidly growing and a slowly growing form Also in these cases the letter *a* on Figs 1 and 3 indicates that the strain on blood agar had the same appearance as the strain from which it was isolated the letter *b* indicates that the colony morphology was only temporarily altered and the letter *c* that the change in the colony morphology was constant for more than five subcultures

In a single case E_{10} (Fig 1) a substrain was included in the material on the basis of another criterion This strain was isolated by chance from an agar stab with F_1 A blood agar plate was inoculated from the stab culture and five successive subcultures were made from single colonies before test pieces were prepared At no time could this strain be distinguished from E_1 on blood agar but the strain showed an altered resistance to ionizing radiation

Prior to the irradiations to determine the inactivation curves all strains were subcultured at least five times on blood agar from a single colony to ensure a uniform population on irradiation Doses between 1 2 and 7 5 Mrad were used for these irradiations

Test pieces for the irradiations were prepared by culture of the bacteria on blood agar plates at 36–37° C for one to five days depending on the rate of growth of the strain in question The cultures were then scraped from the plates suspended in serum broth and dried as uniform drops (0 01 or 0 02 ml) on polyethylene foil at room temperature and in air (4) The number of viable units per test piece before and after irradiation was determined by using suspensions in phosphate buffer saline (pH 7 38) employing conventional dilution and counting techniques All counts were made on blood agar

As the inactivation curves for Str faecium were non linear the comparison between the radiation resistance of the strains was made on the basis of the inactivation curves For practical reasons there had to be an interval of about 24 hours between irradiation and the first determination of viable units on the irradiated test pieces The inactivation curves were in all cases drawn on the basis of this determination

All irradiations were performed at Risø the Research Establishment of the Danish Atomic Energy Commission either in the Co^{60} plant or in the electron accelerator facility The dose in the Co^{60} plant was given with an accuracy of + 2 per cent and in ... an accuracy of + 5 per cent[1]

...ed could all be identified as *Streptococcus fae*... ...haracteristics of the strains were studied by the methods for enterococcal diagnosis usually employed in the department (9 15)

RESULTS

Radiation Resistance of the Strains

In the *first experimental series*, the resistance to ionizing radiation was determined for *Str faecium* E_1 and 17 substrains of this strain Fig 1 illustrates the relationship between these 18 strains and Fig 2

[1] The authors wish to thank the *Danish Atomic Energy Commission* and the staffs of the Co^{60} plant and the electron accelerator facility

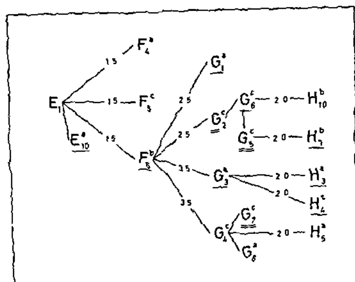


Fig 1

The relationship between the strains in the first experimental series The figures *between the strain designations indicate the irradiation dose in Mrad received by* the preceding strain prior to the isolation of the following substrain Where no

... resistant to radiation than F6 Strains marked with *a* had colonies with the same appearance as the strain from which they were isolated strains marked with *b* had temporarily changed the appearance of their colonies and strains marked with *c* had retained a change in colony appearance through at least five successive subcultures

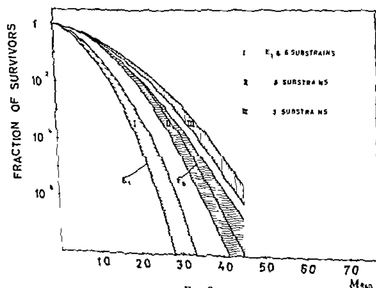


Fig 2

Inactivation curves for *Str faecium* E1 and 17 substrains of it

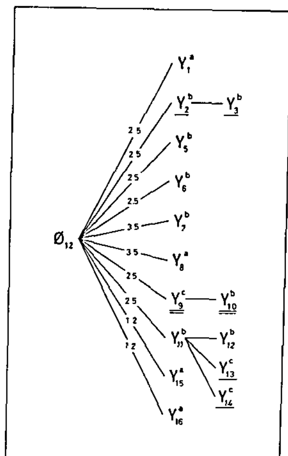


Fig 3

The relationship between the strains in the second experimental series The figures between the strain designations indicate the irradiation dose in Mrad received by the strain before the new substrains were isolated Where no figure is given the substrain was isolated without intervening irradiation Strains without an underlining had the same resistance to ionizing radiation as $Ø_{12}$ strains with one underlining had a lesser resistance than $Ø_{12}$ and strains with two underlinings had a greater resistance than $Ø_{12}$ (For the marks *a* *b* and *c* see also text to Fig 1)

shows the inactivation curves on the basis of the number of viable units 24 hours after irradiation

Five of the substrains examined had a higher resistance than the strain from which they were isolated Eight substrains had unchanged resistance, and four had a lower resistance than the strain from which they were isolated

Seven of the 17 substrains were representatives of the majority of bacteria, which had not altered their appearance on blood agar plates after exposure to ionizing radiation In six of these seven substrains, the resistance was unchanged in comparison with that of the strain from which they were isolated (strains marked with *a* on Fig 1) The exception was E_{10}, the criterion for the selection of which was in fact that it showed a change in radiation resistance (see Fig 1 and remarks under material and methods)

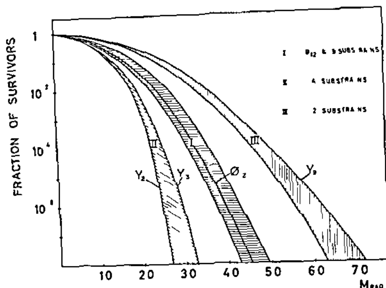


Fig 3
Inactivation curves for *Str faecium* $Ø_{12}$ and 15 substrains from this strain

In the *second experimental series* the resistance to ionizing radiation was determined for *Str faecium* $Ø_{12}$ and 15 substrains isolated from it Fig 3 illustrates the relationship between these 16 strains, and Fig 4 shows the inactivation curves on the basis of the number of viable units 24 hours after irradiation

Three of the 15 substrains were less resistant to irradiation than the strain from which they were isolated and one substrain was more resistant Compared with $Ø_{12}$ nine substrains had the same resistance as $Ø_{12}$ four had a lower resistance and two a higher resistance On selection four of the 15 substrains showed the same kind of colony as $Ø_{12}$ These four substrains all had the same resistance to ionizing radiation as $Ø_{12}$

In both experimental series changes in resistance to ionizing radiation were most common among those strains which retained altered appearance of their colonies through at least five successive subcultures (strains marked with c on Figs 1 and 3) The relations between the criteria for selection and the changes in resistance to ionizing radiation are shown in Table 1

Post Irradiation Effect

The number of viable units per irradiated test piece decreased in the course of the days after the irradiation and this reduction in the number of viable organisms was not the same for the various strains (Figs 5 and 6) In some cases it was observed that this post irradiation effect could vary with changes in the mode of packaging the test pieces in the

polyethylene foil as well as with changes in the effectiveness of the drying A systematic study is now in progress on these points

The irradiated test pieces were stored in the dark at room temperature before the determination of viable units Under these circum stances, no case of reactivation of the bacteria was observed, but as mentioned, the culturing took place at the earliest 24 hours after irradiation, so that nothing is known as to the number of viable units per test piece prior to this

TABLE 1

Relations between the Criteria for Selection and the Changes in Resistance to Ionizing Radiation

Resistance to ionizing radiation	Number of substrains			
	In total	The appearance of colonies on blood agar plates		
		a Unchanged	b Temporarily changed	c Change constant for five successive subcultures
Unchanged	19	10	7	2
Decreased	7	0	3	4
Increased	6	(1)*	1	4
In total	32	10+(1)	11	10

* Substrain E_{10} the criterion for selection of this particular strain was a change in radiation resistance

Cultural and Biochemical Characteristics

In the substrains isolated, no correlation was demonstrated between resistance to ionizing radiation and the cultural and biochemical characteristics examined

When subcultures in broth were examined in the microscope, either after Gram staining or using phase contrast microscopy of unstained preparations, a number of the strains were found to show clumping of diplococci and chain formations, some of the bacteria in these aggregates were large and very refractile, but these phenomena were not correlated with the radiation resistance of the strains

After growth for 1–4 days on blood agar at 37° C all the strains developed α haemolytic colonies All the strains satisfied Sherman's enterococcal criteria, with the exception of the test for growth at pH 9 6, which is not employed in the department for the diagnoses of enterococci All the strains were resistant to methylene blue (0 1 per cent), and grew on 40 per cent bile agar, they broke down arginin and fermented mannitol, arabinose, glucose, maltose lactose trehalose, salicin and esculin None of the strains grew on a solid medium containing potassium tellurite in a concentration of 1 2500 or fermented sorbitol, melizitose, raffinose, inulin and glycogen

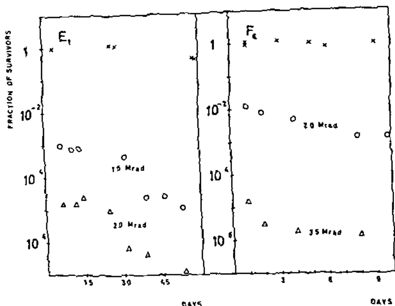


Fig 5

Examples from the first experimental series of the variation in the number
viable units per test piece during the days after the irradiation

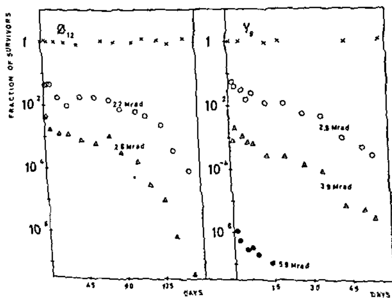


Fig 6

Examples from the second experimental series of the variation in the number
viable units per test piece during the days after the irradiation

In the case of F_6 (Fig 1) the ability to ferment sucrose was lost, and this change was characteristic for all substrains derived from F_6

DISCUSSION

It has been known since 1956 that some micrococci are very resistant to ionizing radiation (1) In the dried state, *Str faecium* can also show a resistance to ionizing radiation which is very great in comparison with the resistance shown by most vegetative bacteria (2)

As already mentioned, micro-organisms have been proved to be able to alter their resistance to ionizing radiation as a result of exposure to irradiation The alterations in resistance previously demonstrated in various bacteria has not implied that these mutants could be expected to be of importance in sterilization by ionizing radiation, as in spite of the alteration, the resistance of the mutants was still very small in comparison with the resistance of bacterial spores

However, laboratory strains of *Str faecium* as well as faecium strains isolated from dust and dirt, in some cases have such great resistance to ionizing radiation, that these bacteria are of the greatest importance when determination of doses for radiation sterilization of dry plastic material is requested It must be of practical significance, therefore, to know that also these very resistant strains, as shown in the present study, can change in resistance as a result of irradiation The most radiation-resistant substrains in the present material have a resistance which is of the same order of magnitude as the resistance of *Micrococcus radiodurans* in the dried state (4) Might it be that *Anderson*'s well-known, very resistant strain R_1 of M radiodurans (6, 7) is a mutant, which has originated from an unknown mother strain of micrococci or from one of the other strains in Anderson's collection of radiodurans strains found in canned meat?

The criteria for selection of the strains comprising the present material were simple and quite arbitrary The aim of the study was to isolate a number of mutants with different inactivation curves, if such mutants actually arose during the irradiation For this purpose, it was found to be convenient to select colonies which in one way or another were different from the majority On the other hand it was not decisive that the change in the appearance of the colony should be stable Changes in resistance to ionizing radiation were also found among colonies which regained the appearance of the mother colony after a single or only a few subcultures Changes in resistance were most common however, among those strains which retained the altered appearance of their colonies through at least five subcultures

If ionizing radiation is to be employed for the purpose of sterilization on an industrial scale, it must be important that precautions be taken to limit the spread of resistant strains from irradiation plants In connection with the industrial use of ionizing radiation, it should be men-

tioned that where the aim of the irradiation is not sterilization but selective reduction of the number of bacteria in a foodstuff (11), for example to improve its keeping power, or to remove a particular undesirable bacterium, the power of the radiation to induce mutations could involve more serious risks than increased radiation resistance in surviving micro organisms Such irradiation by means of a dose which is very small in comparison with the dose required for sterilization must presumably be able to induce the formation of numerous different mutations among the surviving micro organisms As a result, the possibility must be anticipated that unexpected characteristics might appear, *e g* pathogenicity, in organisms which could be regarded as relatively harmless prior to irradiation (13) It is not possible to make a reasonable estimate of the magnitude of such a risk on the basis of the present knowledge

The post irradiation effect demonstrated in dried faecium strains in the present study, may be associated with similar phenomena in dried bacterial spores and dried grain It has been demonstrated previously that the radiation resistance of spores of *Bacillus megaterium* (14, 18) and *Bacillus subtilis* (16, 17) in the dried state can depend on oxygen tension during and *after* irradiation and also on the water content of the spores and on the temperature during and *after* irradiation Similar observations have been made with the irradiation of dried maize seed (10) and dried seed of Himalaya barley (5)

An after effect of radiation with a considerable fall in the number of viable units per irradiated test piece has been observed on two occasions on examining the resistance of dried staphylococcal strains (3) so that the phenomenon is perhaps widespread as assumed by Webb and co workers (18)

The near relationship between the faecium strains examined in the present material together with the pronounced difference between some of the strains in resistance to gamma and electron radiation, make these substrains a promising experimental object for further studies on the after effects of irradiation

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SUMMARY

Following irradiation of two air dried strains of *Streptococcus faecium* with doses between 1 2 and 3 5 Mrad in the electron accelerator facility or Co^{60} plant a number of substrains were selected Some of these substrains had a lower resistance to ionizing radiation than the mother strain some had unchanged resistance and some had a greater resistance than the strain from which they were isolated The most resistant substrains had a resistance of the same order of magnitude as *M radiodurans*

In the case of F_6 (Fig 1) the ability to ferment sucrose was lost, and this change was characteristic for all substrains derived from F_6

DISCUSSION

It has been known since 1956 that some micrococci are very resistant to ionizing radiation (1) In the dried state, *Str faecium* can also show a resistance to ionizing radiation which is very great in comparison with the resistance shown by most vegetative bacteria (2)

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However, laboratory strains of *Str faecium* as well as faecium strains isolated from dust and dirt, in some cases have such great resistance to ionizing radiation, that these bacteria are of the greatest importance when determination of doses for radiation sterilization of dry plastic material is requested It must be of practical significance, therefore, to know that also these very resistant strains, as shown in the present study, can change in resistance as a result of irradiation The most radiation-resistant substrains in the present material have a resistance which is of the same order of magnitude as the resistance of *Micrococcus radiodurans* in the dried state (4) Might it be that *Anderson*'s well-known, very resistant strain R_1 of M radiodurans (6, 7) is a mutant, which has originated from an unknown mother strain of micrococci or from one of the other strains in Anderson's collection of radiodurans strains found in canned meat?

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INVESTIGATIONS OF SOME EFFECTS OF HUMAN SALIVA ON INFLUENZA VIRUS

I *Haemagglutination Inhibition and Neutralization*

By

GUNNAR ROLLA

Received 14 ix 64

The effect of saliva on virus has been investigated earlier (2, 7, 8, 11) *Harmon et al* tested pooled expectorate from patients suffering from chronic bronchitis for neutralizing effect against influenza virus *in ovo* (11) A neutralizing factor was found in the supernatant obtained by addition of 9 volumes of saline to 1 volume of expectorate followed by homogenization in a Waring blendor and subsequent centrifugation at 10,000 × g for 15 min The neutralizing factor was resistant to RDE treatment but could be destroyed by trypsin Corresponding serum titrations were not reported

Krueger could not demonstrate any neutralizing effect of human saliva against A/PR 8 influenza virus by mouse infectivity tests (8)

Jungeblut (7) found no evidence for the presence of neutralizing activity against *Columbia SK* virus in saliva and *Bloch* (2), using infectivity titrations in monkeys, failed to demonstrate any such activity against mumps virus

[illegible]

Whereas the presence of a neutralizing activity in saliva is thus rather inconclusive, several investigators have reported that saliva contains inhibitors of the haemagglutinins of influenza virus (5, 11, 12)

Francis & Minuse demonstrated haemagglutination inhibition of heated virus and infective virus with saliva samples which had been centrifuged at 2000-2500 r p m for 30 minutes (5)

Sellsam et al collected pilocarpin stimulated saliva and demonstrated inhibition of influenza virus haemagglutinins by parotid and sublingual saliva (12)

The strongest activity was exhibited by sublingual saliva The haem-

The number of viable units per irradiated test piece decreased during the days after the irradiation This post-irradiation effect was not the same for the different substrains The after effect appeared to vary with the effectiveness of the packaging and the water-content of the micro organisms

No correlation has been demonstrated between the resistance to ionizing radiation and the other characteristics known in the case of the substrains isolated

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Prior to HI titrations normal inhibitors were destroyed by treatment with cholera filtrate according to the method of *van der Veen & Mulder* (14)

One volume of serum was mixed with five volumes of cholera filtrate (N V Philips Duphar) and incubated over night at 37° C followed by heating for one hour at 56° C. Penicillin and streptomycin had been added to the cholera filtrate The samples were then absorbed with 10 per cent of red cells for one hour at 4° C

Virus The strains of influenza virus used in the experiments are found in the ... Centre A/PR 8 ... kidney tissue ... are pure egg

Tissue Culture

The kidneys were removed from 18 days old chick embryos trypsinized and suspended in tubes Each tube had 10^6 cells in 1 ml of Hanks solution containing 0 1 per cent yeast extract (Difco) 0 5 per cent lactalbumin hydrolysate (NBCo) and 20 per cent calf serum The medium contained 100 I U penicillin G and 0 01 mg of streptomycin per ml The medium used after inoculation of virus contained only 2 per cent calf serum

The medium was changed every 24 hours during the growth period Before inoculation with virus the mon layers were washed three times with Hanks solution

Infectivity Titrations in Eggs

10 fold dilutions of virus were inoculated into embryonated eggs 12 days old using 7 8 eggs per dilution The eggs were then incubated for 48 or 72 hours and then examined for haemagglutinins in the allantoic fluid HA titres of 5 or more were considered positive ID_{50} were calculated according to the method of *Reed and Muench* and were expressed as $\log_{10}$

Infectivity Titrations in Tissue Cultures

... racks The medium

RESULTS

Haemagglutination Inhibition Activity of Sublingual and Parotid Saliva against some Influenza Viruses

The inhibiting effect of parotid and sublingual saliva on the haemagglutination activity of different influenza viruses is presented in Table 1 The inhibition was effective in all tests performed The inhibitory effect of sublingual saliva was usually stronger than that obtained with parotid saliva In some cases (i e B Lee and B Taiwan) the difference between the two samples was so pronounced that ...

... and serological classification

Similar results have been obtained by *Seltsan et al* in experiments with PR 8 and swine influenza virus (12)

Treatment of Red Cells with Saliva

The effect of saliva in HI titrations could have been caused by an influence of the receptors on the surface of the erythrocytes In order

agglutination inhibition of influenza virus caused by human saliva is thus well established. Further investigations on salivary inhibitors, however, have been performed with preparations of ovine and bovine sublingual glands. These inhibitors are macromolecules of glycoprotein nature (3, 9).

The significance of the unspecific, haemagglutination inhibition exhibited by some biological fluids and its relation to neutralization of infection is mostly unknown. The publications in this field have mainly dealt with the nature of cell receptors, rather than with the rôle of receptor analogues in the pathogenesis of influenza.

The relationship between haemagglutination inhibition and infectivity neutralization on one hand and virus antibodies in saliva on the other has not been discussed.

MATERIAL AND METHODS

Collection of saliva. Saliva samples were collected from 10 test persons and pooled. The samples were obtained from two sources: some from glandula parotis and others from the combined secretions from glandula sublingualis and glandula submandibularis. The samples will be referred to as '*parotid saliva*' and '*sub lingual saliva*', respectively.

Whole saliva was collected directly from the mouth of the test persons. All samples were unstimulated resting saliva and were collected at 10–12 a.m. The samples from parotis were collected at the aperture of the parotid ducts. The test subjects rinsed the mouth repeatedly with water. The parotid ducts were then blocked with cotton rolls and a saliva ejector placed under the tongue. After ten minutes the cotton rolls were removed and the parotid secretion squeezed into a glass tube.

The samples of "sublingual saliva" were collected by pipettes in the sublingual area. In this case the parotid ducts had been blocked for five minutes with cotton rolls and mechanical appliances before the collection started.

Preparation of saliva for titrations. The samples were cooled immediately in ice water for 15 minutes. They were then centrifuged for 20 minutes at 4000 r.p.m. in a refrigerated centrifuge. The supernatants were used. The sediments showed haemagglutination inhibition activity but were discarded because of content of bacteria and debris. The parotid samples were clear and serous. The sublingual samples were also clear but rather viscous. The samples were mostly used immediately. When storage was required the samples were lyophilized and stored in sealed glass tubes. For egg inoculation and for inoculation of cell cultures saliva was mixed with antibiotics. To 1 ml of saliva was added 100 I.U. of penicillin G (Glaxo), 50 I.U. of mycostatin (Squibb) and 0.01 mg of streptomycin (Glaxo).

Erythrocytes. Red cells were obtained from Plymouth Rock hens by puncture of a vein in the wing. The blood was collected in a glass tube with 10 per cent of a 3.9 per cent dilution of sodium citrate and was then washed three times in phosphate buffered saline PBS (4). The red cells were stored in PBS at 4° C for not longer than five days when used for titration.

Haemagglutination (HA) titrations. HA titrations were performed in WHO 'Perspex' trays. 0.25 ml of a 0.5 per cent suspension of red cells was added to 0.25 ml of 2 fold dilutions of virus in PBS. The HA titre was considered to be the reciprocal value of the highest dilution of virus giving partial agglutination of the red cells. The reading of the titres was done after one hour at room temperature.

Haemagglutination inhibition (HI) titrations were also done in WHO 'Perspex' trays according to *Isaacs et al.* (6). To 0.25 ml of two fold dilutions of inhibitor in PBS was added 0.25 ml of a 0.5 per cent suspension of red cells. 0.25 ml of diluted allantoic fluid containing 4 agglutinating doses (ADS) of virus was then added. The reading of the titre was done after one hour. The highest dilution which caused partial inhibition of haemagglutination was considered as the HI titre.

volumes of sublingual saliva To the controls was added medium with 2 per cent calf serum After five minutes at room temperature eggs were inoculated in the allantoic sac with 0 1 ml of the virus saliva mixture or 0 1 ml of the control mixture The eggs were sealed with wax Eggs inoculated with A strains were incubated for 48 hours at 33° C and those inoculated with B strains for 72 hours The allantoic fluid was then harvested and assayed for haemagglutinins Every dilution step was inoculated in at least six eggs The results in Table 3 give mean values obtained in two experiments set up on two successive days with the same reagents The haemagglutination inhibition was evident whereas the infectivity was unchanged after treatment with saliva Prolonged incubation of virus saliva before inoculation gave the same results

TABLE 3

Relationship between Virus Haemagglutinins and Infectivity in Chick Embryos

Virus antigen	HA units ml	ID_{50} ml
Before addition of "sublingual saliva		
A/PR 8	5120	$10^{5.5}$
A_2/Japan/305/57	3200	$10^{5.0}$
After addition of sublingual saliva		
A/PR 8	20	$10^{\ 2}$
A_2/Japan/305/57	20	$10^{5.2}$

TABLE 4

Relationship between Virus Haemagglutinins and Infectivity in Chick Cell Monolayers

Virus antigen	HA units ml	ID_{50} ml
Before addition of sublingual saliva		
A/PR 8	5120	$10^{3.5}$
A_2/Japan/305/57	3200	$10^{[illegible] 5}$
After addition of "sublingual saliva		
A/PR 8	20	10^{0}
A_2/Japan/305/57	20	10^{0}

Monolayers of chick kidney cells in tubes were then inoculated with 10 fold dilution of virus containing allantoic fluid which had a high infectivity when tested on eggs To the allantoic fluid was added equal volumes of sublingual saliva before inoculation The medium was sucked off and the tubes were inoculated with 0 2 ml of each virus saliva mixture and incubated at room temperature for 15 minutes All tubes were then washed three times with Hanks solution and subsequently 1 ml of medium was added to each tube After 10 days of

to test this possibility, 0 5 ml of packed red cells were mixed with 2 ml of undiluted, centrifuged sublingual saliva and incubated for 60, 120 and 180 minutes, respectively The erythrocytes were then washed twice with saline and finally suspended to the usual 0 5 per cent suspension in PBS for use in HA-titrations The HA-titres were compared to the titres obtained with erythrocytes not treated with saliva The results in Table 2 showed no significant effect of the treatment

TABLE 1

HI Tests with Sublingual and Parotid Saliva against 4 ADS of Influenza Viruses

Virus antigen	Parotid saliva HI titre	Sublingual saliva HI titre
A/Swine (USA 1930)	320	320
NDV	160	320
B/Lee (New York 1940)	4	2048
B/Malaya/4/58	160	2500
B/Norway/1/59	1280	5000
B/Denmark/1/59	160	1280
B/Utah/2/59	160	1280
B/Tokyo/M27/61	40	80
B/Taiwan/4/62	4	2048
A/PR 8 (Puerto Rico 1934)	4	1024
A_1/Sweden/3/50	160	160
A_1/India/31/55	160	640
A_1/Denver/1/57	40	80
A_1/Eire/1/57	80	1280
A_1/Denmark/2/57	640	10000
A_2/Singapore/1/57	40	320
A_2/Japan/305/57	4	256
A_2/Norway/1/58	5	40

TABLE 2

Haemagglutination Tests with Red Cells before and after Treatment with Saliva *

Virus antigen	HA titre before treatment with saliva	HA titre after inclubation with saliva for		
		60 min	120 min	180 min
A/PR 8	1280	1280	640	1280
A_2/Japan/305/57	1280	1280	1280	1280
B/Lee	2560	2560	1280	2560
B/Ann Arbor/1/59	640	1280	640	640
B/Taiwan/4/62	2560	2560	1280	2560

* Procedure 0 5 ml of packed red cells were incubated with 2 ml of centrifuged sublingual saliva After incubation the red cells were washed twice in saline A 0 5 per cent suspension of red cells were then used in the HA titrations

Relationship between Haemagglutination Inhibiting Effect and Neutralizing Activity

The effect of human saliva on the virus infectivity was investigated To 10 fold dilutions of virus-containing allantoic fluid was added equal

salivary effect on the virus particle The experiments in Table 3 and Table 4 were performed to test any correlation between haemagglutination inhibition and infectivity neutralization No such correlation was found by use of *in ovo* tests

The infective dose causing 50 per cent infection (ID_{50}) in the eggs showed no difference after addition of saliva to the virus dilutions whereas the HA titre was greatly affected As the inhibition by saliva to the red cell-virus system is known to be of a temporary character (12) release of infective virus particles can be expected some time after the inoculation in eggs This virus may infect the allantoic cells even if an inhibitor was acting at the time of inoculation

A host virus system which allowed removal of unadsorbed virus particles after inoculation was considered suitable for demonstration of a possible neutralization by saliva The chick kidney cell influenza virus system was tried The time allowed for adsorption to the chick cells was found essential Too long adsorption time may allow release of virus particles bound to inhibitor Pilot experiments showed that 15 minutes at room temperature was a suitable time With an adsorption time of 25 minutes or more no neutralizing effect by saliva was evident

Our experiments show thus that saliva exercises a neutralizing effect in the influenza virus-chick kidney cell system

SUMMARY

1 "Parotid and sublingual saliva inhibited the haemagglutinins of the A A_1 A and B influenza viruses tested

2 No neutralization could be demonstrated by infectivity titrations *in ovo*

3 Neutralization could be demonstrated by infectivity titrations in cell cultures The time allowed for adsorption was found to be essential in these experiments

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incubation the tubes were tested for haemagglutinins and ID_{50}/ml was calculated The experiments demonstrated a neutralizing activity of human saliva in the virus-chick cell system (Table 4) Pilot experiments had shown that an adsorption time of 15 minutes before washing of the cells was suitable to demonstrate the neutralizing activity of saliva With an adsorption time of 25 minutes or more no neutralizing activity was evident

DISCUSSION

In the experiments described it would have been desirable to use the sterile gland secretions collected directly from cannulated salivary ducts, to assure that the haemagglutination inhibitors originated from the salivary glands The practical difficulties with such a sampling procedure, however, made it rather difficult to obtain sufficiently large quantities of saliva Further investigations concerning the inhibiting qualities of different pure salivary gland secretions are in progress *As described above the saliva investigated in our experiments* was collected after rinsing of the mouth The samples were immediately cooled down to 5° C Preliminary experiments had shown that the haemagglutination inhibitors were rapidly inactivated at 37° C In spite of the sampling technique we consider that the inhibition against virus is due to substances which are secreted by the salivary glands This view is based on the following

a) haemagglutination-inhibitors with corresponding activity have been extracted directly from human (1), ovine (9) and bovine (3) sublingual glands *in vitro*
b) *The composition of purified preparations of haemagglutination* inhibitors from ovine and bovine gland extracts has been reported Substances of similar composition are present in the human salivary secretions (10)
c) *Umemoto* has demonstrated HI-activity in germfree human saliva collected by sterile technique (13)

The experiments presented in Table 1 showed that sublingual saliva and parotid saliva inhibited the haemagglutinins of all influenza viruses tested This is in agreement with the findings of *Francis et al* who demonstrated an inhibitory effect of human saliva on PR 8 and swine influenza *Sublingual* saliva *had the highest activity in our tests* The reason for this may be a higher concentration of the same inhibitor in sublingual saliva than in parotid saliva It is also possible that different inhibitors are present in these secretions

The possibility that the haemagglutination inhibition of saliva is caused by enzymatic or chemical destruction of the receptors on the erythrocyte surface was tested The results given in Table 2 show, however, that saliva had no detectable effect on the erythrocytes in our test system It is thus obvious that the inhibitory activity is due to a

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PRODUCTION OF LARGE QUANTITIES OF ENTEROTOXIN B AND OTHER STAPHYLOCOCCAL TOXINS ON SOLID MEDIA

By

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Received 17 ix 64

Over the past few decades various methods have been devised for producing optimum quantities of enterotoxin (*Surgella* 1947 *Casman* 1958 and 1963 A *Bergdoll* 1962) and other staphylococcal toxins (*Elek* 1959) The problem arises in the testing of the toxin forming capacity of isolated staphylococci and in the production of a basic material for purifying and studying various toxins and enzymes If only a few toxins are to be studied precipitation methods can be used for concentration (*Bergdoll* 1959 and others) Large quantities of concentrated preparations with complete toxin spectra can be obtained interalia by evaporating the whole culture supernate (*Hallander* 1963) by means of vacuum dialysis (*von Hofsten et al* 1960) or by adding cross linked dextrans and then separating in a basket centrifuge (*Flodin* 1962) A disadvantage of concentration methods of this type is that they require simple dialysable media and this is not always compatible with satisfactory formation of toxin High grade dialysable media can be obtained by means of vacuum dialysis (von *Hofsten et al* 1960) but this is a time-consuming procedure If only small quantities of toxin are needed the bacteria can be cultured directly in a d als s c [illegible]

[illegible]

MATERIALS AND METHODS

Stra n The S e [illegible]

[illegible] ca t i usi n broth (D fco) pH 7 4 to which was added 1 5 per cent Bacto agar (Difco) to f rm a solid med um

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TABLE 1

Number of S. aureus per ml in Cultures Grown in Three Modifications on Two Different Media 24 Hours 37° C

	Number of bacteria ml	
	Media according to Casman + 2% proteinhydrolysate × 10^9	Heart infusion broth 10^9
Liquid medium without agitation	7.0	7.0
Liquid medium with agitation	8.2	9.0
Cellophane plate	7.2	7.3

TABLE 2

Culture on Heart Infusion Broth Incubation at 37° C for 24 Hours

	Toxin titres		
	Solid medium with cellophane	Liquid medium without agitation	Liquid medium with agitation
Alfa haemolysin	1/3200	<1/10	1/320
Delta haemolysin	1/320	<1/10	1/40
Lipase	1/160	<1/10	<1/10
Fibrinolysin	1/40	1/10	1/10
Enterotoxin B	1/10	<1/10	<1/10
DNAse	1/80	<1/10	1/10
Alkaline phosphatase	*277F*	*40F*	*20F*
Hyaluronidase	1/4000	1/4000	1/8000

TABLE 3

Culture on Medium According to Casman modified by Adding 2 per Cent Proteinhydrolysate Incubation at 37° C for 24 Hours

	Toxin titres			
	Solid medium		Liquid medium without agitation	Liquid medium with agitation
	With cellophane	Without cellophane		
Alpha haemolysin	1/2560	1/80	1/80	1/320
Delta haemolysin	1/320	1/20	<1/10	1/40
Lipase	1/160	1/20	<1/10	1/20
Fibrinolysin	1/20	<1/10	1/40	1/40
Enterotoxin B	1/320	<1/10	<1/10	1/20
DNAse	<1/10	<1/10	<1/10	<1/10
Alkaline phosphatase	124F	15E	15F	100F
Hyaluronidase	1/8000	<1/1000	1/4000	1/4000

The toxin and enzyme titres obtained after centrifugation appear in Table 2 (heart infusion broth) and 3 (modified Casman medium). Regarding most of the substances investigated the toxin yield per bacterium was far greater on cellophane plates than in liquid medium. Exceptions were hyaluronidase and fibrinolysin, alkaline phosphatase

2) Semisynthetic medium for enterotoxin production according to Casman (*Casman* 1958), containing acid hydrolysed casein (Difco) vitamins cysteine tryptophan iron citrate magnesium sulphate, and sodium acetate pH 7 2 7 4 modified by adding 2 per cent protein hydrolysate (*Bergdoll* 1962) To obtain a solid medium 1 5 per cent Bacto agar (Difco) was added

Culture 1) Bacteria were cultured on a Petri dish (diameter 14 cm) covered with autoclaved cellophane For inoculation bacteria from a blood agar plate were suspended in 5 ml of phosphate buffered saline The density of bacteria was measured in a Beckman Colorimeter Model C (green filter) Usually an extinction of 0 6 was used The suspension was poured onto the plate After varying periods at 37° C the plate was harvested with 10 ml of phosphate buffered saline and centrifuged for 15 minutes at 4 500 r p m Before centrifugation the bacteria were counted as follows 0 1 ml of the bacterial suspension was diluted in 9 9 ml of detergent (0 05 per cent Atlas G) and shaken for 5 minutes with glass beads on a vibrator The bacteria were then counted in a Burker chamber (depth 0 01 mm)

2) Bacteria were cultured on fluid medium 5 ml of bacterial suspension prepared as under 1) was inoculated into 100 ml of fluid medium in a 500 ml Ehrlenmeyer flask After varying periods at 37° C the bacteria were counted and centrifuged as under 1)

3) The bacteria were cultured on fluid medium as under 2) but with agitation on a rotary shaker

Determination of toxin and enzyme titres The supernates obtained after centrifugation were titrated for the presence of alpha haemolysin delta haemolysin lipase fibrinolysin (staphylokinase) enterotoxin B DNAse alkaline phosphatase hyaluronidase In order to express the relative toxin production all the suspensions were diluted to the same bacterial concentration

Alpha haemolysin delta haemolysin lipase fibrinolysin (staphylokinase) alkaline phosphatase and hyaluronidase were determined as described in a previous paper (*Hallander* 1963)

Enterotoxin B The enterotoxin B titre was determined as the greatest dilution still giving a precipitation line in gel diffusion (*Wadsworth* 1957) together with a 'purified antienterotoxin diluted 1/10 The antienterotoxin was obtained by immunizing rabbits with enterotoxin purified by passing through Sephadex G 100 and through a cation exchanger Cellex p (to be published) The serum thus obtained was as 'pure as that supplied by courtesy of Dr *M S Bergdoll* Chicago The same serum was used althrough the investigation

DNAse As a medium 0 3 per cent desoxyribonucleic acid (sperm) + 2 per cent agar in 0 01 M TRIS buffer pH 8 0 was used Plates with basins were poured using matrices for basin plate technique according to Ouchterlony (*Ouchterlony* 1958) The samples were allowed to drip into the basins and the plates were left to stand at 37° C After 5 hours 1 per cent aqueous solution of methyl green was poured onto the plates These were then left for 30 secs and washed with aq dest In the presence of DNAse a clear zone appeared round the basins after a couple of hours at room temperature (*Osowiecki et al* 1963)

Routine examination of enterotoxin formation The bacteria were taken from the blod a saline to an absorption of 0 6 filter—) and inoculated onto a e enterotoxin titre was determined as ab ve

RESULTS

Staph aureus S 6 was cultured in three modifications as described under Materials and Methods on two different media—heart infusion broth and a special enterotoxin medium

Table 1 shows the yield of bacteria per ml after 24 hours culture With these results as a basis the suspensions were diluted to the same bacterial concentration—in this case $7\ 0 \times 10^{10}$ bacteria per ml—before estimation of toxin activity

enterotoxin after 24 hours of incubation at 37° C (to be published separately)

TABLE 5

Inoculates of Different Strength on Cellophane Plate (Modified Casman Medium) Incubation 37° C 18 Hours

Extinction (colorimeter)	Titre enterotoxin B
0 05	1/80
0 1	1/80
0 2	1/80
0 4	1/80
0 6	1/320
0 8	1/320
1 2	1/320
1 4	1/320

DISCUSSION

For some reason cultures of *Staphylococcus aureus* on solid media covered with cellophane gave a better toxin production per bacteria after 24 hours of incubation than cultures in corresponding liquid medium, even when the latter was incubated with agitation The addition of 1 5 per cent agar to the medium in the plate technique may be of some significance, for it is a well known fact that addition of agar at magnitudes of 0 1 0 8 per cent, so-called semisolid agar, promotes toxin formation (*Elek* 1959) However, in the present case it does not seem very likely that the agar as such, consisting as it does of calcium and magnesium salts of polysaccharide sulphate esters, can diffuse through the cellophane memb[illegible]
the [illegible]
cant [illegible]
to a [illegible] ygen supply Another explanation of course is that the maximum of toxin formation occurs later in liquid than on solid medium covered with cellophane and for that reason is unregistered

Presumably the cellophane in this technique prevented extracellular products from diffusing down into the agar This contention is supported by the fact that much smaller quantities were obtained without cellophane Relevant to this is *McLean's* (1937) demonstration that bacteria cultured in cellophane sacs immersed in a medium gave better yields than such cultured directly in corresponding dialysable parts of the medium He suggested that the cellophane might prevent the entrance of inhibitors

The purpose of this work, however, was to solve a practical problem and the theoretical aspects were not thoroughly investigated in this study

The technique with cellophane covered agar plates proved to be simple and well suited to the routine diagnosis of enterotoxin-forming

on Casman medium Plates without the cellophane membrane gave a poor yield throughout (Table 3, column 2) Cultures in liquid medium gave a somewhat better yield with agitation On modified Casman medium the titre of enterotoxin B for example was 1/320 on cellophane plates, < 1/10 on liquid medium and 1/20 on liquid medium with agitation

A comparison between the two media revealed interesting differences Bacteria cultured on medium according to Casman released 30 times as much enterotoxin as those on heart infusion broth On the other hand the yield of DNAse and alkaline phosphatase was somewhat smaller on this medium Regarding the other toxins production was about the same on either medium

TABLE 4
Cultures Incubated for Different Periods of Time on Cellophane Plates (Modified Casman Medium)

	Toxin titres		
	Incubation time 24 hours	Incubation time 48 hours	Incubation time 2 hours
Alpha haemolysin	1/1280	1/160	1/40
Delta-haemolysin	1/320	1/80	1/80
Lipase	1/80	1/40	1/20
Fibrinolysin	1/20	<1/10	<1/10
serum con	1/400	1/800	1/1600–1/3200
Enterotoxin B			
serum 1/10	1/200–1/400	1/400	1/800–1/1600
DNAse	<1/10	<1/10	<1/10
Alkaline phosphatase	100E	138I	150F
Hyaluronidase	1/4000	1/2000	1/1000

Table 4 shows the significance of incubation time After 72 hours the enterotoxin titres were four times as high as after 24 hours However, all the other activities, except alkaline phosphatase, decreased considerably during the same period Alpha-haemolysin for example fell from 1/1280 to 1/40 The use of concentrated antienterotoxin raised the titre one step above that in which serum diluted 1/10 was used Incubation of cellophane plates for 72 hours and titration against concentrated antienterotoxin gave titres of max 1/3200 with the serum used

Since the original aim of the investigation was to devise a method for demonstrating the presence of enterotoxin-producing staphylococci only, the importance of inoculum strength on modified Casman-medium was also studied The results appear in Table 5 Optimum toxinproduction was already obtained at a colorimetric inoculum extinction of 0 6

Of approximately 200 strains isolated from hospital cases and cultured on cellophane plates ca 10 per cent could be shown to form

enterotoxin after 24 hours of incubation at 37° C (to be published separately)

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staphylococci A suitable medium was that described by *Casman* with the addition of 2 per cent protein hydrolysate For routine diagnosis an incubation time of 24 hours appeared to be sufficient Toxin production was sufficiently high to make concentration unnecessary The *antienterotoxin used in a dilution of 1/10 was found to give few non* specific reactions

Accurate comparisons between the yields of enterotoxin by the methods described above and by other methods of toxin production (*Bergdoll* 1962, *Casman* 1963) were not possible on account of differences in the measuring methods used

By varying the composition of the medium it should be possible to selectively promote the production of other extracellular products The incubation time is also most important, since several activities decrease if incubated for more than 24 hours The fact that enterotoxin increases may be connected with the higher heat stability of this substance

The technique gives a good basic material for separation studies since the primary toxin concentrations are high Further, as a result of the cellophane, the problem of the dialysable substrates is avoided a great advantage if the material is to be concentrated

SUMMARY

Cultures of Staphylococcus aureus on solid media covered with cellophane formed far greater quantities of toxin after 24 hours of incubation than those cultured on corresponding liquid medium The method was specially adapted for routine examination of the capacity of isolated staphylococci to produce enterotoxin

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GENETIC FACTORS DETERMINING COMPETENCE IN TRANSFORMATION OF *NEISSERIA MENINGITIDIS*

I A *Permanent Loss of Competence*

By

K Jyssum and S Lie

Received 21 ix 64

Nutritionally deficient mutants of *Neisseria meningitidis* may be transformed by DNA Several nutritional markers have been examined with regard to genetic linkage in transformation (8)

When mixed cultures of incompetent auxotrophs of *Neisseria meningitidis* are plated on basal agar media, mixed colonies may appear, in which various pairs of auxotrophs grow by a mutual cross feeding However, no genetic recombination can be detected in mixed colonies of incompetent bacteria It has been postulated that such an establishment of mutual cross-feeding is only one expression of a general tendency of meningococcal cultures to develop interdependence between individual units The difficulties met in attempts to isolate auxotrophs by the penicillin method has been discussed in the light of this hypothesis (7)

Genetic recombinations occur when mixed cultures of auxotrophs of *Neisseria meningitidis* are plated on the surface of agar media provided that at least one of the participating auxotrophs is competent of transformation (8) The experimental evidence has been taken to indicate that such recombination is due to transformations of competent cells present in mixed colonies which grow by cross-feeding until sufficient concentration of extracellular DNA is reached

In previous communications the terms competent and incompetent meningococci have been extensively used (7, 8) This paper describes some differences between these two variants Competence may be discussed from at least two points of view Most authors have been engaged in the phenotypic expression of competence, *i e* the period in which an actual cell is competent and the conditions which favour the development of competence Less attention has been paid to the other aspect, competence as a potential property of the cell, in other words, the genetic background of competence In this communication attempts have been made to find a genetic background for the difference between the competent and the incompetent variant of the meningococcus The

problem of physiological competence will be treated in another publication (9)

MATERIALS AND METHODS

The methodology and experimental manipulations used in this investigation were analogous with those previously described (7 8) Some additional technical procedures or modifications have been described in the experimental section

RESULTS

Loss of Competence in some *Strains of Meningococci*

Very early in the course of these investigations it was observed that certain strains of *Neisseria meningitidis* rapidly lost their competence of transformation during serial transfers This seems to be a general observation by those working with transformation of this species (1 2) This type of loss does not seem to be a finding from other transformation systems although low transformable variants may often be found among microbes which are usually highly transformable

TABLE 1

Loss of Competence in some Meningococcal Strains

Strain no	Serogroup	Transformation frequence at the start	Loss of competence in competent clones		
			Un tested	Competent units	Loss of competence per cent
M 1	Group B	+++	1140	1119	1 93
M 2	Group B	+++	500	498	0 40
M 3	Type X	++	200	92	54
M 4	Group B	+++	400	393	1 75
M 5	Group C	+	200	0	>99 5
M 6	Type X	++	500	172	65 6
M 7	Type X	++	200	71	64 5
M 8	Type X	++	100	91	9
M 9	Group C	+	200	1	99 5
M 10	Type X	+	108	0	>99 1

+++ = 10^{-3} 10^{-4} ++ = 10^{-4} 10^{-5} + = 10^{-5} 10^{-6}

All determinations were performed with DNA carrying a str r marker Frequency of transformati … to the tech
nique … f competence
after prea … streaked on
plates which … es were pre
incubated f … in order to permit a potential competence to be d
veloped and any …
m … ed ver t an
… u ed

Experiments were first performed to find out whether the type of competence loss observed is a common feature among meningococci or confined only to certain strains

Ten strains which had originally been isolated from the cerebrospinal fluid of patients in Norway (6) were tested for competence of transformation The system was a transformation from str s to str r DNA

for these transformations was prepared from the strains M1 Prot str r and from M6 Prot str r An ampoule from a culture of each strain lyophilized immediately after the isolation, was opened, and the content spread on blood agar plates for single colony isolation At the same time 5 ml of brain heart infusion broth (BH) was inoculated and grown to the middle logarithmic growth phase Conventional transformation was performed with the BH culture as the recipient strain (8) Single colonies were next picked from the blood agar plates and checked for competence by streaking as described in the legend to Table 1 When a competent clone had been found, this was further examined for loss of competence as recorded in the table

All the strains tested were at the start competent according to the conventional criteria, although the frequency of transformation per recipient cell differed greatly It is also clear, however, that all ten strains throw off incompetent descendants When considering the loss of competence in a competent clone the three strains M1, M2 and M4 point themselves out by having a significantly smaller loss than the other strains In the remaining strains the loss is in fact so high that competence is very rapidly lost upon subculture

Further studies were mainly performed with the strain M1 and its mutants as representative for the group with a comparatively small loss of competence The strain M6 and its mutants have been used in some experiments representing the group with a very high loss of competence

The Frequency of the Competence Loss

Although all ten strains have been shown to contain non-competent variants in any population sufficiently heavy, the frequency with which such negative variants appear, differs from one strain to another When considering the more stable strains M1, M2 and M4 it is seen that competence is absent in 0 4 to 1 93 per cent of the colony-forming units In the other strains competence is absent from a far larger part In three strains more than 99 per cent of the individual units in a competent clone were scored as negative

From the data presented in Table 1 it is also seen that a connection may exist between the frequency with which competence is lost and the serotype of the strain The significance of this observation remains to be found

Considering the diplococcal nature of the meningococci it might be assumed that the appearance of an incompetent clone in a competent population were due to a segregation and not to a mutation In order to explore this possibility more than 1000 single competent clones from each of M1 Prot and its mutant Aux 12 his have been examined for their loss of competence A more stable line, with regard to competence loss, has *not* been found in any instance

The behaviour of the competent and the incompetent variant was also examined in mixed culture technique. Mixed cultures of the competent and the incompetent variants of the strains Aux 12 his and Aux 14 thr were prepared and plated on blood agar plates. From picked single colonies three types could be distinguished with regard to competence. One was a pure, negative line, the second was a pure line with loss corresponding to that found in the competent starting strain. The third type, obviously representing the mixed colony (7) had a loss of approximately 50 per cent. This type of loss was found to represent a segregation into the two former types.

The Permanence of the Competence Loss

Competence negative variants of the strains M1 and M6 have been maintained in this laboratory for more than two years without reappearance of competence. A number of experiments have also been performed with these strains to explore the stability of the incompetent variants.

At first attempts were made to demonstrate a spontaneous reversion to competence in the negative variants. Heavy populations were scored for the presence of any competent variants (back mutants) by the following two systems employing the incompetent variants of several auxotrophs. a) Auxotrophs were treated with DNA from a str-r mutant of the same auxotroph according to the standard technique used in transformations (8). Various exposure times and times for phenotypic expression were tried in each experiment. Any str-r colonies which appeared were tested for competence of transformation to prototrophy with DNA from a prototroph. b) In the reverse system DNA from a prototroph without a str-r marker was used. Any colonies which appeared on the basal plates were tested for competence by means of DNA carrying a str-r marker. Although these systems are highly selective no reversion to competence has been observed.

In a following series of experiments the populations of incompetent cells underwent various treatments prior to performance of screening for a presence of competent cells. These treatments may be summed up as follows:

1. Treatment with UV light.
2. Treatment with acridine dyes.
3. Treatment with DNA from competent variants.
4. Treatment with filtrates and ultrasonic extracts from cultures of competent variants.
5. Treatment with a period of aerobic growth followed by non aerobic incubation according to *Goodgal & Herriott* (3).
6. Serial passage in mice.

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Serial cultures of Aux 12 his were run in 5 ml of BH Each culture was grown to the stationary phase. Then 0 1 ml was used to inoculate another culture Aliquots from each culture were spread on blood agar plates and single colonies were scored for competence In one typical experiment 51 negative colonies were found among 200 scored from the third subculture with DNase In the control series without DNase only 3 out of 200 colonies scored were negative The experiments clearly showed that the presence of DNase during serial transfers significantly increases the number of competence negative clones recovered

The experiments with DNase are on the whole taken to indicate that DNase, while without influence on the change from competence to incompetence, protects any negative variants formed as well as their descendants against some eliminating mechanism At least in the strain M1 the competent variant has a survival value which is eliminated or reduced when DNase is present in the system

Attempts to Demonstrate Infectivity

One property found among several cytoplasmatic particles in *E coli* is the infectivity (4) Many attempts have been made to demonstrate similar properties of the hypothetic genetic determinant of competence in meningococci

The experimental approach followed several lines In the first experiments two parallel series of mixed cultures of auxotrophs were run, one with DNase added and the other without The auxotrophs were grown to the exponential growth phase in complete media (BH) and mixed in equal proportions in fresh, complete medium The cultures were incubated for 20 hours at 37° without shaking Next they were plated in dilutions on selective media which permitted growth of the "recipient strain" Single colonies were picked and checked for growth requirements, for evidence of mixed population (7), and for competence At least 1000 colonies with the properties of the "recipient strain" *i e* the strain which at the start was incompetent, were examined for competence in each experiment The data from some experiments in this technique have been recorded in Table 2

The data from the experiments in which DNase had been added are conclusive since a transfer of competence has never been observed although several thousand colonies have *been examined after mixed* culture technique Apparently, however, the competence may be transferred to an incompetent auxotroph when DNase is removed from the system In the experiments recorded in Table 2 competence seems to have been introduced into Aux 6 arg, Aux 5 phe as well as into Aux 12 his This effect, however, may most likely be considered the result of an "*in vivo* transformation followed by a segregation (8).

Several experiments were performed to explore the segregation of genetic markers in mixed cultures in which a transformation was

The latter experiments were performed with the mucin technique (11) The bacteria were recovered from heart blood of the mice in each passage

It has not been possible to demonstrate a presence of competent cells in these experiments

The Effect of Acridin Dyes, UV Light and DNase on the Competence Loss

In a following series of experiments the influence of several treatments on the frequency of loss of competence was tested

At first cells were grown in complete medium (BH) to the exponential phase, and then inoculated into fresh, complete medium with and without 2 µg acriflavine per ml These cultures were incubated overnight without shaking at 37° The cells were then centrifuged, resuspended in saline, diluted and spread to give single colonies on blood agar plates Competence was tested by streaking as described in the legend to Table 1 At least 500 colonies were tested in each experiment In several experiments with the test strains Prot M1, Aux 12 his and Aux 14 thr the acriflavine series showed a loss in 1 2 per cent, while the control series showed a loss in 1 6 per cent, of the colonies tested

Unfortunately, the concentration of acriflavine cannot be increased above 3 µg per ml due to a very high toxicity of this chemical to meningococci This concentration is very much lower than that used in experiments with *E coli* which is usually 20 µg acriflavine per ml (4)

In another experimental series the acriflavine treatment was combined with an irradiation by UV light according to a technique previously used for *E coli* (4) No increased loss of competence could be demonstrated

These results seem to indicate that the hypothetical genetic factor which determines competence in meningococci, with regard to its behaviour towards treatment with acridine dyes and UV light does not behave in the manner previously described for autonomous, episomal factors in *E coli*

The influence of DNase on the frequency of the competence loss was also explored In these experiments 5 ml of BH with DNase was inoculated from a competent clone of the strain to be tested A control tube was also inoculated in which the enzyme had been omitted The cultures were grown to the late logarithmic growth phase and spread on blood agar plates One hundred colonies were examined for competence in each experiment In one series of experiments 400 colonies from the DNase series showed a loss of competence in 8 while the control series without DNase had a loss in 4, of the 400 tested These experiments indicate that the presence of DNase in batch cultures does not influence the frequency of competence loss

The presence of DNase during serial transfers was next examined

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The results from the mixed culture experiments are in agreement with the assumption that the competent strain takes up genetic material from the incompetent strain (as well as from competent strains) when they occur in mixed colonies (8) The transformed competent strain segregates recombinants during further growth Thus the progeny from the mixed colony contains the total genetic material in all sorts of combinations

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TABLE 2

Introduction of Competence into some Auxotrophs of Neisseria meningitidis *by Mixed Culture Technique*

Exper. no.	Auxotroph tested as recipient (cp$^-$ strain)	Auxotroph used as donor (cp$^+$ strain)	Recovery of cp$^+$ recipient cells: With DNase	Recovery of cp$^+$ recipient cells: No DNase
1	his pro	thr	—	—
2	arg	his	—	+
3	phe	his	—	+
4	his	thr	—	+
5	arg ilva	thr	—	—

More than 1000 cols. with nutritional markers of the recipient auxotroph were tested for competence in each experiment. The technique has been described in the text.

TABLE 3

Recombinations between the Competence Factor and Markers from Incompetent Auxotrophs in Mixed Cultures

Exper. no.	Incompetent auxotroph (cp$^-$ strain)	Competent auxotroph (cp$^+$ strain)	Recombinant types recovered from mixed colonies
1	his pro	thr	his cp pro cp$^+$ Prot cp$^+$
2	arg	his	arg cp Prot cp
3	arg ilva	his	Prot cp
4	arg ilva	thr	Prot cp

The technique has been described in the text.

In the case of the Aux 15 his pro a positive competence may be found among the progeny of the mixed colony combined with his pro$^+$, his$^+$pro as well as with his pro. The competence is lost from these recombinants with a frequency corresponding to that found in the original donor of competence Aux 14 thr (0.5 to 1.5 per cent). An interesting observation in this experiment was the finding that the recombinant type his$^+$pro cp$^+$ was far more frequent than the type his pro$^+$cp$^+$ where cp$^+$ indicates a positive competence.

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Another observation which argue against the acceptance of the competence factor as a conventional chromosomal locus is the apparent permanence of the loss A mutation in a genetic locus might, unless it is a deletion, be expected to mutate back Many experiments have been made to demonstrate such back mutation to competence in *meningo* cocci, but without success Clearly, reversion to competence must be an extremely rare event in these strains, if it ever happens Provisionally, the observations are taken to indicate that the loss of the cp⁺ character is a permanent one

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BRIEF REPORT

SPLENECTOMY AND SERUM PROTEINS IN THE GUINEA PIG

By Stig Bryde Andersen[1], Inger Grunnet and Jørgen Clausen[1]

Formation of normal gamma globulins seems to take place predominantly in the plasmocytic cell lines. These cells occur in the bone marrow, liver, spleen, thymus and lymphoid tissue. Recent experiments in the rat suggest that normal gamma globulins are produced to a greater extent by the spleen than by lymphoid tissue elsewhere in the body (*Andersen & Bierring* 1964). The purpose of the present investigation was to study in the guinea pig the significance of the spleen for the production of normal gamma globulin. Total splenectomy was performed in eleven adult male guinea pigs (weight about 400 gram, age about three months) and a sham operation was performed in ten animals of the same weight and age. The surgery was carried out under ether anesthesia. The animals recovered quickly and both groups gained weight at the same rate. They were killed by bleeding through an incision of the abdominal aorta about 60 days after the operation. Serum was studied with paper electrophoresis (*Laurell, Laurell & Skoog* 1956), agar gel microelectrophoresis (*Wieme* 1959) and immunoelectrophoresis (*Scheidegger* 1955).

TABLE 1

Average Results of Paper Electrophoresis in Adult Guinea Pigs after Splenectomy

	Sham operated controls (N = 10)	Splenectomy (N = 11)
Total Serum Protein (Kjeldahl)	4.66 ± 0.16	4.75 ± 0.50
Serum Albumin	2.80 ± 0.15	2.85 ± 0.30
α_1 globulin	0.28 ± 0.014	0.29 ± 0.025
α_2 globulin	0.73 ± 0.06	0.77 ± 0.11
β globulin	0.35 ± 0.03	0.30 ± 0.04
γ globulin	0.51 ± 0.08	0.55 ± 0.12

Results are given in grams per 100 ml with the standard deviation.
N = number of guinea pigs in each group.

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seven splenectomized and in four control guinea pigs β_2 I globulin was decreased in six splenectomized and in two control guinea pigs α_2 III globulin was decreased in seven splenectomized and in two sham operated guinea pigs The pattern of the remaining protein fractions did not deviate

TABLE 2

Average Results with the Standard Deviation (in gram/100 ml) of Agar Gel Micro Electrophoresis in Guinea Pigs after Splenectomy

	Sham-operated controls (N = 10)	Splenectomy (N = 11)	Mobility range
Total Serum Protein (Kjeldahl)	4 66 ± 0 16	4 75 ± 0 50	
Serum Albumin	2 70 ± 0 30	2 75 ± 0 50	0 78 – 0 95
α_1 globulin	0 61 ± 0 13	0 52 ± 0 12	0 67 – 0 78
α_2 globulin	0 62 ± 0 15	0 76 ± 0 16	0 52 – 0 67
β_1 globulin	0 19 ± 0 05	0 22 ± 0 06	0 43 – 0 52
β_2 globulin	0 12 ± 0 08	0 09 ± 0 02	0 29 – 0 43
γ globulin	0 41 ± 0 11	0 41 ± 0 11	–0 07 – 0 29

Mobility range (MR) indicated in relation to dextrane (MR = 0) and to human serum albumin (MR = 1 0)

The present findings indicate, contrary to the findings in the rat, that the spleen *does not seem to play a major role for the production of normal gamma globulin* in the guinea pig This may be explained by the fact that the spleen in the guinea pig is a much smaller organ relatively than in the rat The low production of normal gamma globulin in splenectomized rat may thus be due to removal of a great fraction of the gamma globulin producing tissue The finding that splenectomy does not seem to affect the gamma production in guinea pigs might be explained by the fact that only a little fraction of the gamma globulin producing tissue is removed These findings seem to exclude that the spleen produces or contains a factor governing the production of normal gamma globulin

A highly significant reduction of the beta globulin fraction was found by paper electrophoresis This agrees with the finding in splenectomized rats (*Andersen & Bierring* 1964) This observation could not be confirmed with agar gel microelectrophoresis but immunoelectrophoresis showed that β_2 I globulin and β_2 II globulin tended to be low in splenectomized guinea pigs It is probable that these two beta proteins one of them corresponding to the γM globulin serum
Recent is formed by lymphocytes (*van Furth* 1964) Our findings may therefore suggest that lymphocytes in the spleen in particular is responsible for the production of γM globulin

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BRIEF REPORT

SELECTIVE DEMONSTRATION OF VASCULAR ENDOTHELIUM BY A MODIFIED METHOD FOR GLUCOSE 6 PHOSPHATASE

By Jerzy Kazimierczak

In 1953 *Chiquoine* (1) using the Gomori lead technic and in 1956 *Wachstein & Meisel* (2) using a modification of this same technic demonstrated the presence of glucose-6-phosphatase in renal tubular and hepatic parenchymal cells Since *Wachstein* (3) observed occasional staining of capillaries in kidney sections examined for glucose-6 phosphatase an attempt was made in the present study to accentuate the reaction in the capillaries and to diminish or abolish the reaction in other types of cells

Pieces of human kidney liver heart intestine pancrease and stomach obtained at autopsy and pieces of the same organs from decapitated mice were quenched in isopentane cooled by solid CO_2 sectioned in a cryostat at 6 μ mounted on cover slips and allowed to dry in air for 10 min The dried sections were fixed for 1 to 15

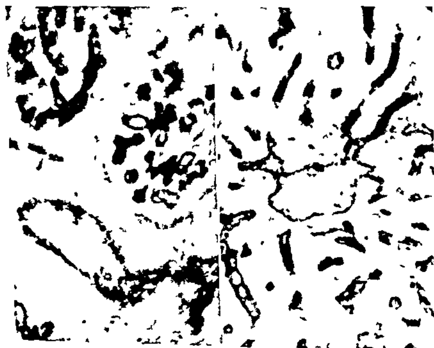


Fig 1 Fig 2

Fig 1 Human renal cortex from autopsy of new born child Prominent staining reaction in the glomerular capillaries and arterial endothelium × 455

Fig 2 Mouse liver Fresh frozen section Only the endothelium of sinusoids and central vein are stained × 455

Received 14 i 65 from the Rheumatic Research Laboratory the University Institute of Pathological Anatomy (Head Prof G Teilum M D) Copenhagen

hrs in cold neutral 5 per cent formalin containing 1 per cent of $CaCl_2$ and rinsed in 20 mM tris maleate buffer pH 5 They were then incubated for 1 to 8 hrs at 32° C in a medium containing glucose-6 phosphate (potassium or sodium salt) 5 mM tris maleate buffer pH 5 20 mM and lead nitrate 4 mM at pH 4 8 to 5 2 adjusted with 0 1N HCl if necessary

After incubation the sections were rinsed in distilled water sulfided rinsed again and counterstained with carmalum The final reaction product was observed exclusively in the vascular endothelium in all tissues examined (Figs 1 and 2) Particularly strong reactions were observed in the capillary loops of the human glomeruli although in badly autolysed organs the results were difficult to interpret The reaction was stronger in the small than in larger arteries Fixation for less than 1 hour very often produced a reaction in the renal tubular and hepatic parenchymal cells of fresh material Prolonging fixation for 15 hrs at room temperature completely abolished reaction in the vascular endothelium This same effect was also observed after adding 10 mM sodium fluoride to the incubating medium The substitution of β glycerophosphate adenosine mono or triphosphate for glucose-6-phosphate in the incubating medium did not reveal any reaction product in the vascular endothelium

On the basis of these observations it would appear that the enzyme in the vascular endothelium responsible for the liberation of phosphate from glucose-6 phosphate differs form that found by Chiquoine in renal tubular and hepatic parenchymal cells in that, the former is more resistant to the action of fixatives and operates at a lower pH However further studies are needed to confirm this interpretation

References 1 *Chiquoine A D* J Histochem Cytochem *1* 429 1953 —2 *Wachstein M & Meisel E* Ibid *4* 592 1956 —3 *Wachstein M* Ibid *3* 246 1955

Acta path et microbiol scandinav 63 321 332, 1965

The Neuropathological Laboratory
[illegible]

CEREBRAL EMBOLI AS A RESULT OF "MUTE" JUVENILE ENDOCARDITIS

By

EDITH RESKE-NIELSEN, K SVENDSEN
and H SOGAARD

Received 26 vi 64

Endocarditis in children is a rare disease The case to be cited here has several peculiar features For instance, the patient's endocarditis was 'mute', clinically as well as during special examinations At the *post mortem* a large heart infarct was found which had given no symptoms and which did not show up during electrocardiograms There were also old infarcts in the kidneys which had never given physical complaints

Two similar cases have been reported earlier by *Castleman et al* (1955) and *Steen Olsen et al* (1962), respectively, though with a somewhat different etiology

CASE HISTORY

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p 622, fasc 4, vol 62, 1964

Berg K Immunochemical studies of anti Lp(a) sera Acta path et microbiol scandinav in press 1965 a

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there was both psychic and somatic improvement, with the result that the patient was moved to a nursing home where his condition *remained stationary until his* accidental death

Laboratory Tests

The routine laboratory tests presented nothing of particular note Hb per cent 92–118, SR max 12 mm/l h ; red and white cell count normal, *aside from leucocytosis—12700* (5 February 1962) Serum cholesterol and fasting blood sugar did not increase Once, urinalysis showed a trace of albumin (4 November 1957) and a few erythrocytes (5 February 1962), but was otherwise normal Blood cultures or serological tests for rheumatoid disease were not taken Repeated tests of the cerebrospinal liquid showed nothing abnormal Blood pressure was normal

X-Ray Examination

X-Rays of the thorax (*August 19, 1957*) showed an uncertain enlargement of the left atrium of the heart This however, was not rediscovered during a special examination at the cardiological laboratory two days later, at which time the patient's electrocardiogram was also normal An examination a year later presented nothing certainly abnormal X-Rays of the cranium showed nothing abnormal The carotid angiography showed—in agreement with the clinical findings—occlusion in the arteria cerebri media dx , one centimetre from the arteria carotis *interna* (1957), two occlusions, independent of each other, in the arteria cerebri anterior dx (1958), and possible but doubtful, impediments to passage in the horizontal portion of the left arteria cerebri media (December, *1961*) *Vertebral angiography showed nothing ascertainably* abnormal Conclusion the patient had recurring cerebral attacks of unknown origin

Post Mortem

The cor weighed 285 grams Its form was normal, its breadth 10 centimetre, its length 8,5 cm The right ventricle wall was 4 mm thick, the left 16 mm There were no congenital malformation, or signs of pericarditis *The endocardium* was greyish-white with moderate fibrous thickening of the septum and apex The valvula mitralis was pathologically altered, in that excrescences of two types were found Some were quite small *(3–4 mm in diameter) easily removable*, *brittle* excrescences, which left small ulcerations on the valve rims others were firm, fibrous, stalky, broadbased, nonremovable formations The valve lips had a moderate fibrous *thickening while the chordae tendineae* showed no ascertainable pathological alteration Widespread fibrous streaks were seen in the papillary muscles The remaining valves presented nothing abnormal

In examining the myocardium a large (approx 4×5 cm) fibrosis was found in the anterior-wall of the left ventricle, extending into the



Fig 1
Fresh excrescence from valvula mitralis (Haematoxylin eosin)

septum and the apex region of the right ventricle The anterior wall of the left ventricle bulged outward aneurysmatically, and measured 5 mm at that point Several small fibrosis were spread over the rest of the myocardium

Regarding the coronary vessels, in the ramus descendens anterior the lumen was closed off immediately from its origin by an old, re-channelized thrombus The remaining coronary vessels were normal

Several old small infarcts could be seen in both kidneys, but an attempt to find vessel occlusions did not succeed The remaining organs showed acute stasis but were otherwise normal

Microscopy

Re- [illegible]

m [illegible]

so [illegible] and Gram

The cor The excrescences on the mitral valve consisted partly of fresh elements constructed of fibrin, red blood corpuscles, and isolated leucocytes (Fig 1) and partly of older organized processes consisting of finely fibrilled connective tissue with few nuclei and without vessels or inflammatory cells and especially no bacteria were seen (Fig 2) The remaining valves were normal The endocardium was thickened with fibrosis In the myocardium, in addition to the large, nearly acellular fibrosis numerous small intramural fibrous streaks were found The pericardium showed some fibrosis A section from

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the ramus descendens anterior showed the effects of occlusion as a fine fibrillar network with numerous newly-formed vessels The re-channelizations could be seen particularly well at the periphery (Fig 3) Nothing abnormal was found in the remaining vessels Here and there along the vessels were small, presumably reactive lymphocyte infiltrates, but nowhere in the cor bacteriae or granuloma were found

The kidneys Some old infarcts were found but no fresh

The remaining organs presented nothing unusual Of note, however, was the fact that no vascular alterations were found

The post mortem and microscopy showed both fresh and old endocarditis, with infarcts in the cor and kidneys

Macroscopical Examination of the Nervous System

The weight of the brain after fixation was 1120 grams The brain had a completely abnormal external shape Both hemispheres were smaller than normal, but the variance was most pronounced on the *right side*, where a large depression in the fissura Sylvii area was found, *i e* the area supplied by the arteria cerebri media This area measured 10 × 4 cm Its edges were bordered by atrophic gyri, and its lower part consisted of brain tissue which was completely atrophied and covered by thick, dark brown leptomeninges Moreover, the gyrus frontalis superior was completely destroyed and remained only as a narrow border of atrophic tissue, clothed with thick leptomeninges Only 4 cm of gyrus praecentralis near the sulcus interhemisphericus remained, and similarly, only 3 cm of gyrus postcentralis (Fig 4) Cortex atrophy was likewise seen on the base of the right occipital lobe This area measured 3 5 × 4 cm and occupied the whole base the pole excepted

The *left hemisphere* was diffusely atrophied with infarcts in the temporal region

The leptomeninges were blurred all over and thickened at the base of the brain The vessels of the circle of Willis were thin—macroscopically without thrombi or emboli

The cerebrum was cut by coronal sections The enormous d ffe- ence of th [illegible]

siz [illegible]

ior [illegible] examination there were large infarcts in the anterior, media and posterior areas on the right side Cortex and the white substance as well as the anterior third of corpus callosum were totally or partly destroyed Corpus striatum and thalamus were atrophied In the left hemisphere several small cortical infarcts were found taking up parts of gyri and the bordering white substance, especially in the posterior half of the hemisphere The ventricle system was deformed dilated and displaced to the right The walls were dull and coarsely bulged, without exudate As a consequence of the destruc-



Fig 2
Organized verruca from valvula mitralis (Haematoxylin eosin).

Fig 3
Rechannelized thrombus in ramus descendens anterior cordis

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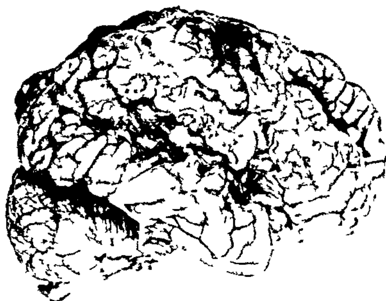


Fig 4
Macrophoto of the brain the right side



Fig 5
Frontal section through the central part of the hemispheres with infarcts

tion of corpus callosum the right anterior horn was only separated towards the surface by a thin membrane (Fig 5)

The brain stem was torqued and the right half atrophied Cerebellum was of normal size, but of abnormal shape In addition to the atrophy the brain stem also showed diffuse bleaching of white substance especially of the motor pathways on the right side, continuing on the left side after the crossing over

Medulla spinalis Leptomeninges were dull and medulla was pale and oedematous with effaced marking on the sections and bleaching of white substance Cauda equina was slightly atrophied

Microscopical Examination of the Nervous System

For microscopy all vessels in circulus Willisi with ramifications were taken out also a representative material from all sections of the cerebrum, the brain stem, cerebellum, and medulla spinalis, and both Gasserian ganglions peripheral nerves and muscles from the upper and lower extremities and the lingua

The following stains were used hematoxylin eosin, van Gieson, toluidine Weil elastin, Mallory orange G

The vessels in circulus Willisi with ramifications There are pathological alterations in all vessels taken out The lumen in both large and small vessels are occluded by thrombi of varying lengths The thrombi consist of a fibrilled network with new capillaries In some places there is also endothel proliferation In the same vessel we find sections which are quite normal thrombosed or with slight endothel-proliferation The walls of the vessels are of a normal structure without inflammation or necrosis (Fig 6 and 7)

Cerebrum In the sections pathological alterations of varying intensity extension and age can be seen in both cortex and the white substance The most severe pathological alterations in cortex are generally restricted to the right hemisphere There are areas (especially frontally and temporally) with total destruction of cortex which has been replaced by loose cicatricial tissue consisting of glia and fibroblasts with numerous thin, proliferating capillaries and sporadic often pigmented—phagocytes From these areas there is a gradual transition to parts with laminar necrosis, mainly including 2nd, 3rd, and 4th layer which are cicatr[illegible] has been preserved with seve[illegible] sis The deep layers are deg[illegible] are observed gyri with nor[illegible] contours, but in which the ganglion cells have disappeared or are calcified There is an increase of both protoplasmatic and fibrilled astrocytes whose ramifications form a fine network sending fibril bundles into [illegible] meningo-cerebral cicatrix The [illegible] and surrounded by several lay[illegible]

Fig 6

An artery with a recent occlusion and a smaller artery with endothel proliferation

Fig 7

A vessel with rechannelization



Fig 8
Laminary cortical malacias

sides these wide-spread pathological alterations there are small wedge shaped infarcts either consisting only of glia or also containing calcified ganglion cells In the preserved parts of cortex there are both diffuse and focal outfall of ganglion cells and varying degrees of degenerative alterations (Fig 8)

In the white substance as well as in cortex there are areas with total destruction and gliosis or diffuse thinning out of the myelin sheaths Round the vessels can be seen phagocytes with break down products and some lymphocytes In the right insula there are several rechannelized vessels in leptomeninges

In the left hemisphere the infarction is specially pronounced in the insula area but the infarcts on this side are younger than on the right side

The brain stem There are diffuse degenerative alterations [illegible] brain stem the [illegible] pathways The d[illegible] normal such a[illegible] [illegible] many contain mostly shrunken cells There is some diffuse gliosis

Cerebellum as in the brain stem we also here find diffuse degenerative alterations but in addition there is an infarct in the white substance, close to nucleus dentatus

Chiasma The myelin sheaths are swollen There is a slight outfall and considerable increase of connective tissue round the vessels

Fig 6
An artery with a recent occlusion and a smaller artery with endothel proliferation

Fig 7
A vessel with rechannelization

had a transient inexplicable febrile period at home This might have been the beginning of the endocarditis At a subsequent special examination there were no signs of any heart disease

Once during the course of the disease albuminuria was found and another time leucocytosis and slight hematuria simultaneously, probably as an expression of the kidney infarcts, but otherwise the patient did not present any signs of kidney diseases, and the laboratory results have been so unnoteworthy that they were not connected with the cerebral attacks

DISCUSSION

Available literature does not describe any cases of "mute' endocarditis in children In our patient the disease manifested itself only through repeated cerebral attacks The patient's severe heart infarct did not give either physical symptoms, cardiologic or radiologic alterations

Repeated cerebral attacks in patients with heart diseases are not infrequent Thus *Hutchinson et al* (1953, 1961) found cerebral attacks in 14 per cent of 323 patients with rheumatic diseases In our patient there are numerous cerebral attacks, but no symptoms from cor or any other organ A similar case has been described by *Castleman et al* (1953) in which a woman of 38 died after numerous cerebral attacks and asymptomatic heart and spleen infarct, whereas the kidney infarcts resulted in slight albuminuria, haematuria and leucocytosis There was endocarditis on the aorta valves in connection with a left sided carcinoma Emboli to cerebrum do not always come from vegetations on a heart valve but can in rare cases be tumour emboli as described by *Steen Olsen et al* (1962) The patient was a woman of 36 who died during a cerebral attack The post mortem disclosed a myxoma in cor with microscopic verified tumour emboli to the brain There were also infarcts in heart kidneys and spleen which had not given any symptoms

Heartinfarction in connection with endocarditis are mentioned by some authors as being rare and by others as being frequent In *Brunson's* work (1953) there is a survey of literature concerning coronary emboli with endocarditis, and he accounts for 9 of his own cases of the same disease which all had macroscopic and/or microscopic emboli with infarcts

At the same time it is pointed out that the electro-cardiographic alterations which are a consequence of the infarcts were observed only in one patient In our patient the electro cardiogram was normal, and despite the large fibrosis in septum and the anterior wall of [illegible] ventri[illegible]

which [illegible]

with [illegible] [illegible] may have extensive myocardial injuries

where there are also *scattered lymphocytes and phagocytes* There is no thrombosis

Leptomeninges round the brain are enormously thickened with proliferating surface cells, numerous fine connective tissue fibrils, thin vessels and accumulation of lymphocytes and phagocytes There are numerous meningocerebral cicatrices Many of the vessels contain rechannelized thrombi, and on the level with the right insula there is also a newly occluded artery with degenerated blood corpuscles, phagocytes filled with fat and with beginning invasion of fibroblasts

Medulla spinalis Leptomeninges are normal Both anterior and posterior roots show a slight outfall of the myelin sheaths The white substance shows status spongiosus, especially along the periphery, but in addition there is considerable outfall and heavy degeneration corresponding to the cortico-spinal pathways and most pronounced on the left side which is in accordance with the fact that the brain injury in the motor region is greatest on the right side The ganglion cells especially in the anterior horns, show outfall and degeneration which is most pronounced in the antero-medial groups and especially in the thoracic spinal cord Some are vacuolated with nuclear displacement, whereas others show different degrees of shrinking and are surrounded by satellites In cauda equina there is a slight outfall of the myelin sheaths, and many of the remaining are swollen and fragmented There are no vascular alterations or thrombi

Right and left ganglion Gasseri There is a slight outfall of ganglion cells with formation of Nageottian nests and degenerative alterations In several places there are lymphocytes especially in the periphery of the ganglion The myelin sheaths in nervus trigeminus and ramifications show outfall and degeneration The Swann cells are proliferating

The peripheral nerves show slight outfall and degeneration and in all the muscles there are a few narrow fibres with nuclei in rows which show a tendency of central displacement, but typical atrophic groups are not seen

Conclusions The macroscopical and microscopical examinations show numerous organized thrombi of various ages in circulus Willisi with ramifications which have caused infarcts The pathological alterations are most pronounced in the right hemisphere There is atrophy of the brain stem, medulla spinalis, and both Gasserian ganglions

Summary of Clinical and Pathological-Anatomical Observations

The patient was a 16 years old boy with numerous cerebral attacks which could not be accounted for as it was not clinically possible to show congenital or acquired disorder of the heart At the post mortem examination was found fresh and old abacterial endocarditis as a cause of the boy's attacks in organs and nervous system

In his anamnesis the mother states that at the age of eight the boy

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University Department of Pathology General Hospital Malmö Sweden

SPLENOMA

By

TH BERGE

Received 27 viii 64

Many different kinds of benign tumours of the spleen have been described The commonest are lymphangioma haemangioma and splenoma the last mentioned tumour occurring exclusively in the spleen Splenomas have also been described under such names as splenadenoma fibrosplenoma fibrosis circumscripta nodular hyperplasia of the spleen and lymphoma of the spleen

According to *Friedreich* (5) the first to report a splenoma was *Rokitansky* (1861)

MATERIAL

Malmo has a *population of 230 000* inhabitants who are served by a single hospital 60 per cent of all *persons dying in* Malmo are necropsied and the necropsy frequency at the hospital *is 98–99 per cent Of th*
more than 10 per cent
distribution peak at the
During the years 19[illegible]7

RESULTS

Fifteen benign tumours of the spleen *were traced* in the necropsy series This group of tumours consisted of 2 cavernous lymphangiomas 2 cavernous haemangiomas 1 cyst (probably an angioma that had undergone cystic *degeneration*) and 10 splenomas The appearance and the distribution of the splenomas among the material are given in the table

DISCUSSION

Splenoma consists of *solitary* or though rarely multiple local hyperplastic processes in the spleen Most of them are the size of a cherry but growths up to 12 cm in *diameter have* been described (1) In two cases (7 14) combined with *hypersplenism* the spleen weighed 1790 gm 750 gm resp In both cases *the spleen* consisted mainly of a large splenoma The gross *appearance of splenomas* differs from that of the rest of the spleen only by its *usually somewhat* paler hue The tumours may be situated centrally as *well as peripherally* in the spleen and have bulging cut surfaces (Figs 1 a 1 b 1 c) Splenomas are rarely encapsulated (10)

SUMMARY

A case history of a 16 years old boy is presented From his 10th to his 16th year, he had had numerous large and small inexplicable occlusions of the brain vessels Not until the post mortem pathologic anatomical examination an acute and chronic verrucal abacterial endocarditis was found which had caused multiple emboli to the brain developing in farctions which had given the patient severe neurological symptoms The infarctions found in the kidneys were possibly responsible to a transient hematuria albuminuria and leucocytosis whereas the mas sive pathological findings in the heart did not give any clinical signs

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4 d Histolog e

5 Ph ladel phia London 1963

6 of the Cardiac

7 sions in

8 Role of Stenosis of the Extracranial Cerebral Arteries Her Majesty s Stationery Office London 1961

Splenomas resemble the spleen also histologically They are made up of follicles red pulp with sinusoids and sometimes abundant fibrous tissue As a rule the picture is dominated by one of these structures and sometimes only one of them is demonstrable

Splenomas are classified according to their appearance as follicular, pulposal fibrous and mixed Microscopically the purely follicular type shows crowded Malphighian bodies with very scanty or no intermediate pulp (Figs 2 a 2 b) The pulposal type is built up of red pulp with sinusoids but also often contains some follicles though fewer and wider apart than in the contiguous normal parenchyma The fibrous type is not generally accepted According to *Vordasini* (12) such processes are only follicular or pulposal splenomas undergoing fibrous degeneration

In the histological examination staining for iron is sometimes very useful According to *Kaufmann* (10), splenomas often contain abundant iron The present 10 cases differed widely in the amount and localization of iron Some tumours contained more, others less iron than the contiguous parenchyma of the spleen In some cases the concentration of the iron was roughly the same in the tumour as in the surrounding parenchyma In 2 cases no iron at all was demonstrable In cases with multiple splenomas the concentration of the iron was higher in the pulposal than in the follicular type

The pathogenesis of the splenomas is unknown Owing to their appearance with normal splenic structures in abnormal proportions these tumours have been regarded as hamartomas originating during embryonal life But *Coe & Drashek* (2) write No convincing evidence of the congenital origin of the nodules have as yet been advanced In 2 of our cases there was reason to assume that the splenoma was younger than the surrounding tissue in that the arteries in the spleen were much more sclerotic than those within the splenoma On the other hand some of the splenomas showed considerable fibrosis and even calcifications This may be explained by the assumption that splen[illegible]

[illegible]

[illegible] and finally fibrous This would be compatible with *Vordasini*s conception of the fibrous splenomas The deposition of iron also argues to a certain extent for such ageing of splenomas (More iron in the pulposal than in the follicular type in cases of multiple splenoma) The high mean age of 76 years (no age was below 67 years) also suggests that splenomas develop late in life

In one case infectious changes were found in the splenoma as well as in the rest of the splenic parenchyma

Co-exist[illegible]

[illegible]

TABLE

Necropsy no	Sex and age in years	Cause of death	Spleen weight in gm	Gross appearance	Microscopical appearance	Occurrence of Ie	
						In splenoma	In rest of spleen
928/57	♀ 81	Hypertension	210	2 cm in diam Purple cut surface	Mainly pulposal Scattered follicles	0	0
481/59	♂ 75	Myocardial infarction	220	Pea sized – Light grey red cut surface	Pulposal	0	(+)
274/60	♀ 79	Myocarditis	175	1 cm in diam Pale red cut surface	Follicular and pulposal	(+)	(+)
863/62	♂ 67	Mb arteriosel cordis Pulm cancer	350	Tangerine sized *Same colour as rest of spleen* (purple)	Mainly pulposal Scattered follicles	+	(+)
335/62	♀ 72	Hypertension Pulm cancer	100	Size of golfball Bright red with fibrous streaks	Pulposal with large fibrous parts + calcifications	++	(+)
902/62	♀ 72	Urethral cancer	200	Plum sized Dark red cut surface	Pulposal	++	(+)
101/63	♂ 86	Polycythaemia	400	4–5 cm in diam Grey red cut surface	Pulposal with large fibrous parts	0	0
512 63	♀ 81	Acute enteritis	120	Hazelnut sized Grey red cut surface	Pulposal with small fibrous streaks	(+)	++
615 63	♂ 72	Arteriosclerosis Pulm cancer	340	Multiple small to hazelnut sized light grey red foci	Some pulposal others follicular	pulposal ++ follicular (+)	++ ++
797 63	♀ 76	Gastric cancer	150	3 cm in diam Dark red cut surface	Mainly pulposal Scattered follicles	+	++

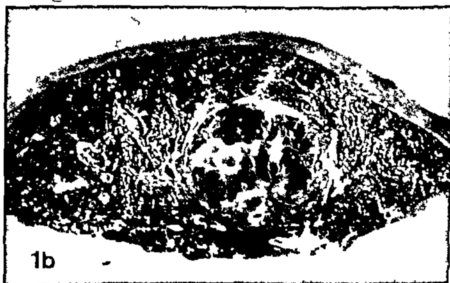


Figs 2a and 2b
Follicular splenoma with sparse Malphighian corpuscles in surrounding splenic parenchyma

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Figs 1a 1b and 1c
Central and peripheral splenoma of pulposal and fibrous type


1a


1b


1c

tructures but in proportions which are different from those in the surounding parenchyma Different types of tumour are usually recog nized namely follicular, pulposal, fibrous and mixed Owing to their appearance splenomas have been regarded as hamartomas by some authors Certain changes suggest that splenomas develop late in life but age more quickly than the neighbouring tissue The descriptions are based on 10 cases of splenoma seen at necropsy of all together 8 114 ases

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higher than in the entire necropsy material, where it was about 40 per cent But the difference hardly allows any valid conclusions

Splenomas have been conceived as intralienal accessory spleens or as accessory-spleen like tumours within the spleen This conception however, appears less feasible since the tumours are usually not en capsulated (10)

Fasanotti (3) believed splenomas to be due to some toxic factor, a view which has not been generally accepted

No reliable figures are available of the frequency of splenomas Of 270 necropsies, *Poscharissky* (13) found what he called "knotige Hyperplasien" in 9 But judging from drawings given in that article, the lesions he saw were angiomas undergoing sclerosis, an assumption also shared by *Lubarsch* (8), who stated that he himself had found similar hyperplastic changes in 18 out of 10,000 necropsies *Lubarsch* distinguishes two types, one in which the tumour bulges only slightly above the surrounding parenchyma and is difficult to identify also histologically, the other, in which the tumour is built up of connective tissue poor in cells and containing thick-walled veins He found that the latter type was often multiple He gives no figures on the relative frequencies of these two types His paper includes no illustrations, but his report of the second type suggests that the tumours described were sclerosed angiomas These previous publications convey no opin ion of the frequency of splenoma, since they consisted of reports (1, 2, 3, 4, 7, 9, 11, 12, 14, 15) of series of at most 6 cases (12) Two of the papers (9, 15) also reported the finding of megakaryocytes and one of them also cells from the myelopoetic and erythropoetic systems thus suggesting that the tumours might have been myelolipomas

Splenomas are probably more common than suggested in the lite rature These tumours are readily overlooked unless the spleen is cut into thin slices Moreover only two cases (7, 14) have been reported where a splenoma caused clinical symptoms (hypersplenism) According to photographs they where of the pulposal type The symptoms disappeared after splenectomy

With these two exceptions it has not been possible so far to ascribe any physiological or pathological function to the splenomas Apparently they have never been diagnosed prior to operation or necropsy Wider knowledge of these tumours may one day contribute to a better understanding of the functions of the spleen

Fruhling & Delage (6) have reported one case of "splenadenoid lymphoblastic sarcoma" which they consider a malignant splenoma

The frequency found in the present material, *i e* one case per 800 necropsies, is probably low

SUMMARY

Splenomas are solitary or multiple, well defined, but not encap sulated, foci situated within the spleen and built up of normal splenic

found wide spread granulomatous lesions of epithelioid cell type in lymph nodes and spleen—together with cardiovascular and glomerulonephritis like lesions—in rabbits after a single intravenous injection of crystalline bovine albumin These granulomatous lesions were reversible and appeared in the immune phase *i e* 12–15 days after the injection The changes gradually declined and disappeared by the 28th day at a time when free antibody was present in the serum *Teilum* 1956 reported that rabbits after several months of immunization with formalin killed cultures of Pfeiffer bacillus showed pronounced granulomatous and necrotizing changes in the spleen and lungs while the kidneys liver adrenals and myocardium showed similar but more scattered lesions He found a correlation between the functional status of mesenchymal cells and the development of abnormal precipitates of protein in the reticulo-endothelial system (amyloid hyalin paramyloid) on the one hand or the formation of epithelioid cells on the other The present paper aims to elucidate the pathogenesis of epithelioid cell formation in the light of this hypothesis

MATERIAL AND METHODS

The animals used were 35 C3H and AKR strain mice which were 2 3 months old of both sexes and 25–30 grs in weight The immunization was carried out with Freund s adjuvant (Bacto adjuvant complete Difco) The dose varied from 0 05–0 2 ml given as weekly subcutaneous injections In a few cases intraperitoneal injec

cases Sud

Sometime

No serolo

RESULTS

Gross examination Clinically most of the animals gradually showed a characteristic picture the severity of which was dependent on the dose of the adjuvant the interval between injections the route of injection and the degree of complicating infection The general condition of the animals as a rule became poorer with decreased activity dullness of the fur and loss of hair The abdomen became larger arched and meteoristic Three animals died as before mentioned probably from anaphylaxis

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GRANULOMATOUS LESIONS IN MICE PRODUCED BY FREUND'S ADJUVANT

Morphogenesis and Phasic Development

By

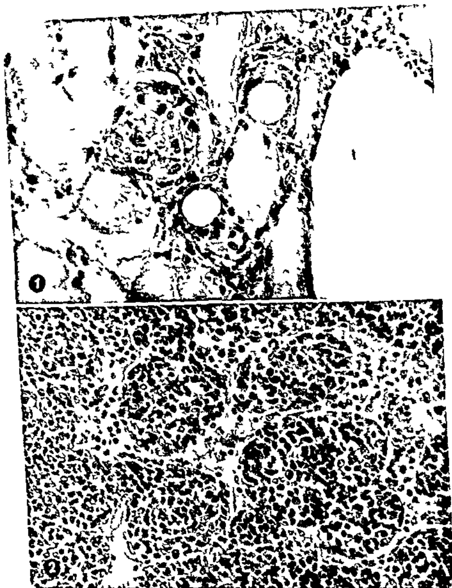
J WANSTRUP and H E CHRISTENSEN

Received 9 ix 64

The development of granulomatous proliferation of cells belonging to the reticulo endothelial system following immunization of various laboratory animals by injections of Freund adjuvant was described, among others, by *Laufer, Tal & Behar* 1959, *Steiner, Langer & Schatz* 1960 and *Rupp, Moore & Schoenberg* 1960 Similarly one of us (*Christensen* 1962) during animal experiments on amyloidosis by the use of Freund adjuvant (*Rothbard & Watson* 1954) confirmed these changes consisting of severe diffuse granulomatous hyperplasia in the subcutaneous tissue, lymph nodes, parenchymatous organs and serous cavities The present work is a continuation of these studies and comprises further investigations on the cytology and morphogenesis, with special reference to a phasic character of granuloma formation (compare the theoretical considerations by *Teilum* cited below)

The reactions of mesenchymal tissue during antigen stimulation and antibody formation have often been the subject of investigation in the last two or three decades Much of our knowledge about this problem has come from Scandinavian workers, particularly *Bing & Plum* 1937, *Bjørneboe & Gormsen* 1943, *Bjørneboe, Gormsen & Lundquist* 1947 and *Fagraeus* 1948, 1958 Correlations between changes in mesenchymal tissue during experimental immunization and the pathology of well-known human diseases or syndromes have been pointed out Among the first, *Klinge* 1929–30 and *Vaubel* 1932 produced experimental lesions comparable to those seen in rheumatic fever and periarteritis nodosa Since then other experimental studies on human material have revealed similar correlations in human diseases (*Rich & Gregory* 1943–46, *Teilum* 1956)

Granulomatous proliferation during stimulation with antigenic substances is also a well-known phenomenon Thus, *Rich* 1952, apparently one of the first, described wide spread granulomatous proliferation of epithelioid cells in spleen and lymph nodes of rabbits, which had received multiple injections of horse serum Similarly *Germuth* 1953



Figs 1 2

Fig 1 Periphery of injection area Striated musculature with well demarcated granulomas built up of reticular cells showing some cytoplasmatic PAS positivity PAS stain × 350

Fig 2 Medullary cords of mesenteric lymph node with marked proliferation of pyroninophil cells and a mixture of greater cells with beginning PAS positivity Several mitoses PAS stain × 350

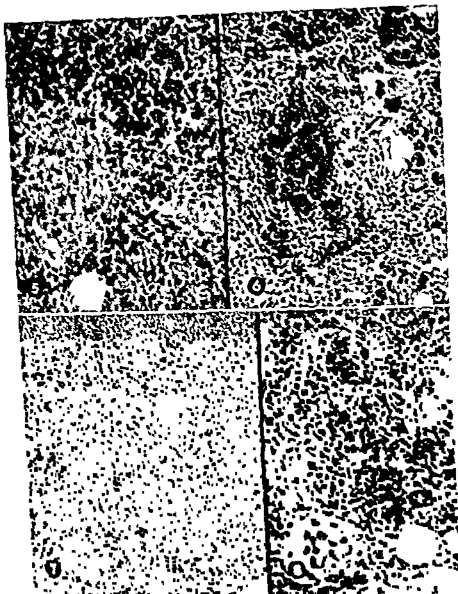
At autopsy marked induration and small cysts containing a viscous, yellowish liquid were observed at the injection site The regional and internal lymph nodes were swollen, but discrete The serosa of the pleural and peritoneal cavities and the mesentery showed a pronounced greyish polypoid proliferation often associated with an exudate in the pleural space and with a more serous fluid in the peritoneal cavity The parenchymatous organs were encapsulated by this proliferating serosa, but the liver and spleen were enlarged In one case a perirenal abscess was found, apparently caused by too deep an injection

Microscopy All animals exhibited varying degrees of generalized pathological changes, consisting of diffuse as well as granulomatous mesenchymal proliferation and hyperplasia An early change was an activation of primitive reticular cells These were large, hypertrophic cells with abundant clear cytoplasm and a round or oval rather large, vesicular nucleus with one or two large pyronino-positive nucleoli (the activated reticulum cell of Marshall and White (*Marshall & White* 1950)) With increasing antigen loading increased reticular hyperplasia was observed in nearly all parenchymatous organs and in the perivascular zones of connective tissue Conspicuous was a pronounced accumulation of mature and especially immature reticular cells with varying degree of cytoplasmatic pyroninophilia Often such cells were found in granulomatous clusters, but also the more diffuse hyperplastic areas resembled confluent granulomata This early or young lesion is termed "pyroninophil granuloma" Perhaps this is still not a "lesion', but a response

The fate of the pyroninophil granuloma was studied in these experiments The cells appeared to become larger with relatively more abundant cytoplasm and decreased pyroninophilia, and—an important observation—cytoplasmatic PAS-positivity appeared Concomitantly the granulomatous lesions become lighter and larger, sometimes showing multinucleated giant cells With increasing PAS-positive material in the cytoplasm Russell bodies were sometimes seen intracellulary or extracellulary, the cells boundaries became less distinct and the cytoplasm appeared vacuolated and cloudy, especially in the center of the granuloma In the periphery of the lesion, lymphocytoid cells or single granulocytes were seen, but neither fibrinoid material nor necrosis was observed Frozen sections from the parenchymatous organs revealed no stainable neutral fat in the granulomas

The "PAS positive granuloma' with extracellular PAS-positive granules and an occasional giant cell was the most advanced granulomatous change observed in the present work, and now it is definitely a lesion,—perhaps provoked by a response Its resemblance to the epithelioid cell granuloma is obvious, and possibly the morphogenesis of the latter is similar

Topography of the lesions The *injection site* showed in all cases wide spread cellular infiltration, with small cystic spaces centrally



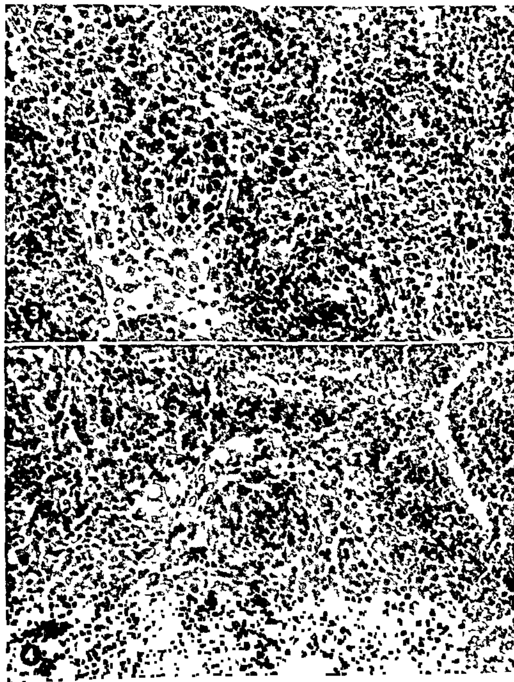
Figs 5–8

Fig 5 Loose medullary granuloma with cytoplasmatic and interstitially PAS positive granula PAS stain × 350

Fig 6 Lymph node with cortical granuloma and PAS positive "giant cells" PAS-stain × 350

Fig 7 Regional lymph node with several light granulomas and darker clusters of pyroninophil cells H E stain × 140

Fig 8 Lymph node showing clear granuloma with cytoplasmatic PAS positivity and clusters of pyroninophil cells probably "granuloma precursors" H E-stain × 220

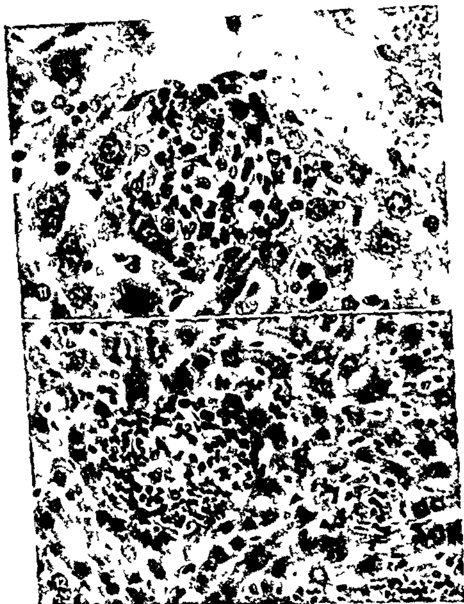


Figs 3–4

Fig 3 Mesenteric lymph node showing medullary granuloma with greater strongly PAS positive cells some with Russell body like inclusions PAS stain × 350

Fig 4 Early PAS positive granuloma in mesenteric lymph node PAS stain × 350

containing a yellowish viscous material, apparently partly originating from the oil in which the antigen was suspended In these central areas there were numerous lipophages Occasionally acute inflammatory changes and vasculitis were present, but these changes were seen only locally in the injection area More peripherally a pronounced perivas-

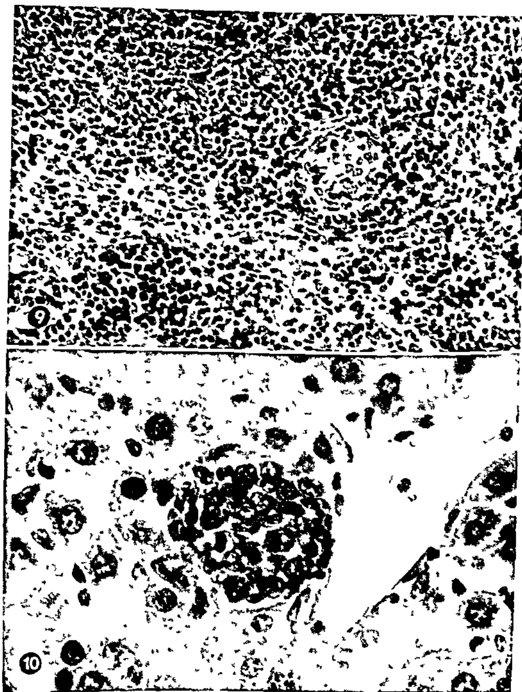


F gs 11 12

F g 11 l er w h l gh er para ascular granuloma show ng some cytoplasmat c I AS posit ty PAS sta n × 560

F g 12 l er w h ntralobulary PAS posit ve granulomas I AS sta n × 440

ul r plasmacellular proliferation was seen and interstitially *in the strat l* musculature circumscribed l ght *and weakly* PAS positive granulomas without accompanying reaction of lipophages or granulocytes were found (Fig 1)



Figs 9–10

Fig 9 Spleen with clear PAS positive granuloma' in perifollicular collar surrounded by clusters of cells showing varying degree of pyroninophilia PAS stain × 350

Fig 10 Liver with proliferating pyronino positive cells in granuloma like paravascular arrangement (i e pyroninophil granuloma) H E stain × 560



Figs 15–16

Fig 15 Kidney with intertubular collection of strongly pyronino positive cells in paravascular position i e "pyron nophil granuloma" H E stain × 350

Fig 16 Kidney showing intertubular collection of pyronino pos tive cells and clusters of greater cells with cytoplasmatic PAS positivity H E stain × 350

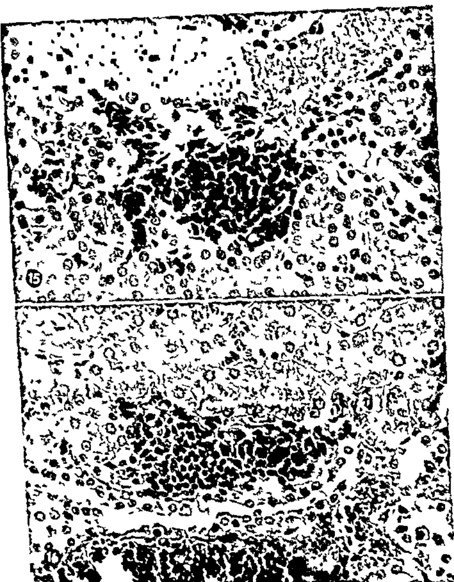


Figs 13 14

Fig 13 E stain × 140

Fig 14 transition between pyronino ma H F stain × 350

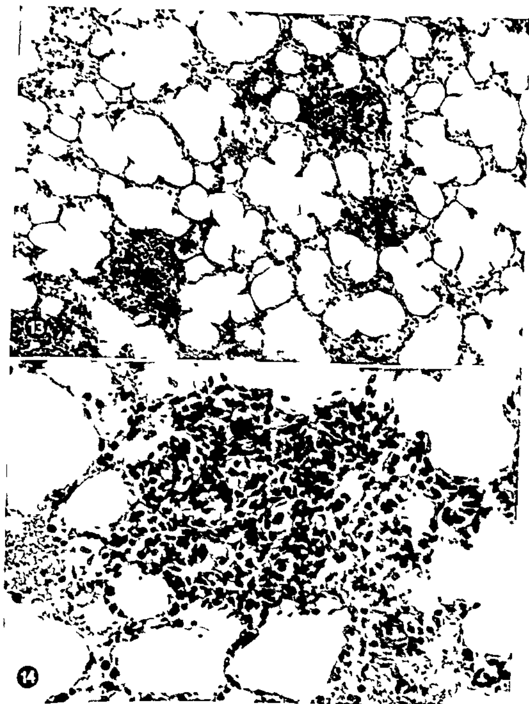
The *lymph nodes* showed varying degrees of hyperplasia and pronounced pyroninophilic cell infiltrations in the medulla and in the perifollicular zones of the cortex distributed either diffusely or in dark cellrich clusters resembling granulomas (pyroninophil granuloma)



Figs 15–16

Fig 15 Kidney with intertubular collection of strongly pyronino positive cells in paravascular position i e "pyroninophil granuloma" H E stain × 350

Fig 16 Kidney showing intertubular collection of *pyronino-positive cells* and clusters of greater cells with cytoplasmatic PAS positivity H E stain × 350



Figs 13 14

Fig 13 Lung with septal granulematous lesions H E stain × 140
Fig 14 Higher magnification of Fig 13 Showing the transition between pyronino phil granuloma and PAS positive granuloma H E stain × 350

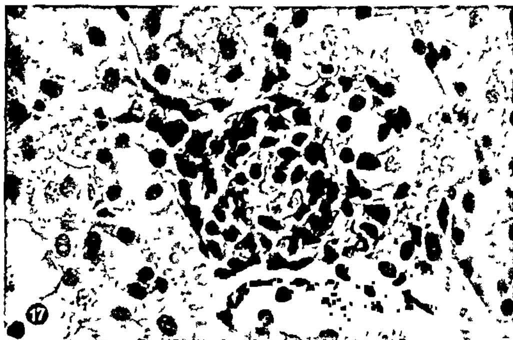
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Figs 15–16

Fig 15 Kidney with intertubular collection of strongly pyronino positive cells in paravascular position *i e* "pyroninophil granuloma" H E-stain × 350

Fig 16 Kidney showing intertubular collection of pyronino positive cells and clusters of greater cells with cytoplasmatic PAS positivity H E stain × 350



Figs 17
Kidney with young 'PAS positive granuloma' H E -stain × 440

These were located both in the medulla, most often in a paravascular position, and in the periphery of the often hypertrophic cortex of the lymphoid follicles Also a gradual transition to lighter cell clusters was observed, sometimes with PAS-positive granules intra- and extracellulary ("PAS-positive granuloma") (Figs 2–8)

The *spleen* was hyperplastic with severe perifollicular accumulation of heavy pyronino-positive reticular cells in both diffuse and granulomatous arrangement Further, there was a varying degree of cytoplasmatic PAS-positivity, and occasionally cells resembling the Kurloff-bodies in the guinea pig were seen Also lighter granulomas in a perifollicular position were observed (Fig 9)

The *liver* showed many especially intralobular granulomas in various
S positivity, i.e both "pyroninophil
" and transitional stages between
nantly a paravascular localization
(Figs 10–12)

Lungs, kidneys and *intestine* also exhibited rather pronounced cellular proliferation, mainly consisting of pyroninophil cells In the alveolar septa of the lungs many hypertrophic PAS-positive reticular cells were found, sometimes as free alveolar cells, but in many cases veritable PAS-positive granulomas were found (Figs 13–14)

In the intertubular zones of the kidney dark clusters of pyroninophil cells in predominantly paravascular position were found, sometimes with a varying mixture of PAS positive cells and a corresponding transi

tion to lighter granulomas (Figs 15–17) No certain granulomatous lesions were found in the glomeruli

Other findings The Freund adjuvant kidney consisting of severe dilatation of the renal tubules (*Rothbard & Watson* 1954), hypercellularity of the glomerular tufts, the dysproteinaemic liver (*Wuketich & Siegmund* 1958) and amyloidosis were occasionally seen in our experiments, but were not further investigated Encephalitis (*Tal, Laufer & Behar* 1958) and other systemic effects of Freund's adjuvant were not investigated

DISCUSSION

The reactions of mesenchymal tissue to diverse noxious stimuli including antigens show definite characteristic cellular phases consisting of hypertrophy, proliferation and pyroninophilia As pyroninophilia reflects increased ribonucleic acid content (*Brachet* 1941) and this again is a sign of increased protein synthesis (*Caspersson* 1941) it is possible in this way to study the morphological criteria of antibody production As stressed by *Fagraeus* 1948 the immature plasmatoid cell types—transitional cells—are most active in this process

In some cases an abnormal change in these reactions occurs apparently due to some disturbance in cellular metabolism, *i e* defective ribo nucleic acid synthesis, toxic influences such as hormonal and drug therapy, mitochondrial damage or other biochemical lesions as a result of cellular hyperfunction, etc The common designation for this pheno menon is here termed "exhaustion effect" The visible effect of this on cell morphology may be the appearence of focal and increasing PAS-positive cytoplasmatic degeneration products or abnormal PAS-positive secretions intracellulary and eventually extracellulary or subsequent cell death and loss of contours Thus, under certain conditions, one may see a characteristic sequence of morphological changes in mesen chymal tissue consisting of hypertrophy—proliferation—pyronino philia—*exhaustion effect*—cytoplasmatic PAS-positivity—reticulosis—abnormal tissue proteins These principal experimental observations have been correlated to a series of clinical disease entities, first of all amyloidosis and they constitute the principles in Teilums "Two phase cellular theory of local secretion' concerning the pathogenesis of amyloidosis (*Teilum* 1964) In the papers *Teilum* 1948 a, 1948 b, 1952, 1956 such abnormal cell reactions were described also in connection with reticular granuloma formation, and it was stressed that both abnormal protein precipitations (amyloid) and epithelioid cell formation were thought to be the result of identical and characteristic cell reactions in mesenchymal tissue

The results of the present experiments speak definitely in favour of this assumption in as much as Freund s adjuvant originally in our laboratory was used in experimental amyloidosis investigations (*Christensen* 1962) Thus it was possible, at the same time, to investigate

the cellular reactions in amyloid and granuloma formation, although amyloid in the present study only exceptionally was seen, apparently because of too weak antigenic stimulation

The step-wise development of the generalized granulomas and their cellular constituents make it apparent that they are the result of principally corresponding abnormal cell reactions as those of experimental amyloidosis The pronounced diffuse and granulomatous pyroninophil proliferation disappears focally and is, after an inversion stage, changed to a PAS-positive phase,—and here the continuous stimulation of the immune apparatus apparently represents the exhaustion effect Simultaneous with the increasing cytoplasmatic PAS-positivity the granulomas become lighter, built up of large, irregular cells Cytoplasmatic and eventually interstitial PAS-positive granules occur (Russel bodies? prehyalin? paramyloid?), and on the whole these early changes become "epithelioid"

In comparison with clinical disease entities and especially *sarcoidosis Boeck*, the investigations here reported may throw light on the development of the granulomatous lesion The observation of the pyroninophil granuloma and its possible fate with gradual development to an epithelioid cell granuloma is especially important in as much as *Bellot* (1962) in a paper concerning the earliest stages of Boecks sarcoid describes lesions of similar character We find the comparison justified, as Bellot describes the earliest granulomatous lesion as a more or less circumscribed, paravascular accumulation of mononuclear lymphoid or plasmatoid cells in the striated musculature with an increasing mixture of larger and lighter epithelioid like cells

Continued morphological and cytological studies of such experimental epithelioid cell granulomas and their fate (hyalinosis, fibrosis etc) will be the subject of further investigation, especially with a view to the possibility of drawing definite comparison to the granulomas of *sarcoidosis Boeck* and other spontaneous granulomas of "sarcoid-type"

SUMMARY

The reported experiments consist of reticular granuloma formation produced by Freund s adjuvant injections into mice

Two types of generalized granulomas are described a young type termed the "pyroninophil granuloma" and later stage termed "PAS-positive granuloma" The changes constitute a two phasic development of the granulomas with transitional stages, and are postulated to be the result of principally similar cellular reactions as those in experimental amyloidosis Such changes reflect the common mesenchymal cell reactions, which *Teilum* 1956 found principal in the formation of amyloid, abnormal protein precipitation and epithelioid cell granuloma

The relation to similar lesions in man, especially Boecks sarcoid and other granuloma formations of "sarcoid-type", is discussed

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PRIMARY FIBROUS HAMARTOMA OF THE HEART

By

SVEN DAHLGREN and BJORN NORDENSTROM

Received 12 ix 64

Tumours of the heart are relatively rare Especially does this apply to primary heart tumours *Strauss & Merliss* (1945) reported that only 8 cases had been communicated to the American Medical Association in the years 1938–42 In the corresponding period 480331 autopsies were according to the statistics performed in America Thus on the basis of this material primary heart tumours occur in only 0 0017 per cent of cases Metastases to the myocardium appears to be somewhat more common and *Prichard* (1951) calculated that about 3 9 per cent of patients with malignant tumours had metastases to the heart

Of the primary tumours in the heart myxoma seems to be the most common and constitutes nearly 50 per cent of these (*Prichard* 1951) These generally proceed from the atrium, and they have, moreover, been diagnosed *in vivo* with cardioangiography (*Kjellberg, Nordenstrom, Rudhe Bjork & Malmstrom* 1962) and with electrokymography (*Ödman & Varions* 1957) In the latter case there was an oscillating tumour in the left atrium When the patient was examined in the forward leaning position the tumour caused a hindrance of flow to the left ventricle, which gave rise to electrokymographic changes as in cases of mitral stenosis The remaining 50 per cent of cases consist of angioma, rhabdomyoma lipoma fibroma, hamartoma and primary sarcoma The fibroma and the fibrous hamartoma are generally referred to one group There appear to be no reliable figures in the literature of the frequency of these forms of tumour In a scrutiny of the literature, however, *Schumann* (1962) did find 18 fibromatous heart tumours in children, published between the years 1927 and 1962 Further, *Freeman* and co workers (1963) have collected 27 cases of intramural ventricular heart fibroma Only a few of these tumours were diagnosed while the patients were alive (*Edlund & Holmdahl* 1957, *Wight* and co workers 1964)

As the development of heart surgery has nowadays made it possible in certain cases to remove by operation tumourous formations localized to the heart itself the diagnosis has for these diseases acquired in-

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Fig 1

a an l b) Frontal and lateral radiographs of the heart with considerable enlargement of the right s de of the organ

creased actuality It is here the authors' intention to give an account of a case of heart tumour in which before the operation it was possible with the help of coronary angiography to obtain roentgenological evidence that the tumour proceeded from the heart wall (*Nordenstrom, Ovenfors & Tornell* 1962) The charting of the vascular conditions in the tumour made it also possible to plan an operative removal of the growth

OBSERVATIONS

A previously, on the whole healthy woman aged 40 years (B D 20 07 08) who on a routine fluoroscopic examination had proved to be suffering from a right sided enlargement of the heart with a total heart volume of 1050 ml During the last year the volume of the heart increased to a total volume of 1330 ml corresponding to a relative volume index of 760 ml/m²

The enlargement of the heart was chiefly due to an enlargement of the right side of the organ (Figs 1 a and b) The right atrium also showed a somewhat bulging outline laterally

The patient complained of fatigue and at times a pressure over the chest and stabbing sensations in the right side On auscultation it was found that the first heart sound was somewhat weak, and that there was a systolic murmur of the 2nd degree over I 3–I 4 sin The blood pressure was 120/80 mm Hg FCG in the states of rest and work was on the whole normal Also spirometric examination showed normal values Heart catheterization with shunt diagnosis with determination of oxygen saturation gave normal findings In vena cava superior and in the right atrium the pressure was found to be up to 6–7 mm Hg The pressure in the right ventricle and the pulmonary artery was normal as was also the stroke volume

Coronary angiography (Figs 2 a and b) showed that the right coronary artery was more strongly developed than the left, in its widest central part it had a diameter of about 6 mm The coronary artery seemed moreover to be displaced to the left by a tumorous formation in the right part of the heart This formation seemed to receive small branch vessels with in part irregular course from the coronary artery For this reason it was assumed that the tumour probably proceeded primarily from the heart wall and in any case not from the pericardium Large parts of the growth however had no roentgenologically demonstrable contrast filled vessels

Owing to the paucity of larger coronary arteries in the tumorous formation and to the fact that it was in process of rapid growth its operative removal was considered indicated

After right sided thoracotomy the pericardium was opened A large tumour proceeding from the lower part of the right atrium and upper part of the right ventricle was disclosed During extracorporeal circulation in combination with hypothermia the tumour was dissected out As the growth engaged the main branch of the right coronary artery over a 4 5 cm long section it was necessary partly to resect this and replace it with a transplant from vena saphena magna

Postoperatively the patient was treated with respirator However it proved difficult to maintain her blood pressure at a normal level and the ECG taken the day after the operation showed that a ventricular rhythm from two foci could be registered firstly a series with low frequency but without signs of bundle branch block The ECG indicated the existence of a current myocardiac process presenting the appearance of an infarct The patient s condition became progressively worse and she died twenty four hours after the operation in a state of circulation insufficiency

The tumour removed during the operation measured about 14 × 9 × 7 cm It was macroscopically well delimited The consistency was rather soft The sectional area was greyish white with myxomatoid spiralled structure The microscopic examination showed that the change consisted of a moderately cell rich fibromatous tumour of hamartoma type It was not possible to show any myofibrils in the tumour The latter contained quite an abundance of thin walled vessels and a coronary arterial branch and a couple of subpericardiac veins were found super



Fig 3

a and b Histopathological section from the fibrous and rather cell rich tumour Note the rather abundant occurrence of both stouter and finer vessels which are found throughout the tumour a) approx × 220 b) approx × 550

On roentgen examination it has sometimes been possible to observe that some part of the heart is enlarged With the aid of cardioangiography furthermore it has sometimes proved possible to diagnose tumours localized to the heart Coronary angiography, on the other

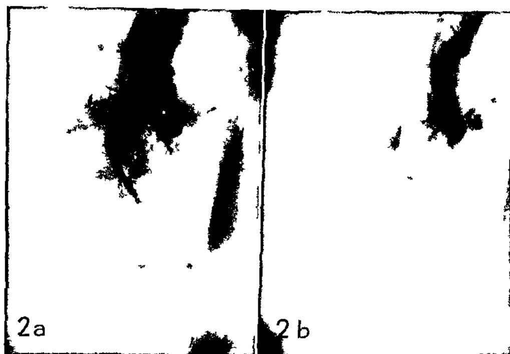


Fig 2

a) and b) Coronary angiography with stereoscopically exposed films The right coronary artery is strongly developed It gives off several branches to the enlarged part of the right side of the heart A number of irregularly running vessels exists in the tumour region chiefly the main trunk of the right coronary artery In large parts of the tumour there are no contrast filled branches of the coronary arteries

ficially embedded in the tumour tissue The delimitation from the pericardium and the surrounding smooth musculature was distinct but there was no actual capsule formation No inflammatory cell infiltration worth mentioning could be demonstrated in the tumour or its surroundings (Figs 3 a and b)

Autopsy revealed a moderate general enlargement of the heart which weighed 450 g The shape was normal but was somewhat contracted over the right atrium at the site of the removed tumour No tumour remains could be demonstrated In order to get a clearer idea of the blood supply to the heart contrast medium was injected into the branches of the coronary vessels Good filling was obtained also of the small branches and no definite thrombus formations could be demonstrated The endocardiac surfaces were normal and no valve changes could be shown

On *microscopic examination* of the myocardium it was observed that the musculature generally was oedematous In the posterior part of the ventricular septum moreover pictures reminiscent of a fresh infarction were observed For the rest the autopsy revealed that there had been a circulatory failure with general acute stasis and pulmonary oedema

DISCUSSION

Primary tumours of the heart seem rather rarely to give rise to clinically observable symptoms In certain cases however disturbances of rhythm peculiar murmurs and sudden pains have been described In the majority of cases the patient has been completely symptom free until he has quite suddenly manifested symptoms of acute circulatory insufficiency

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MORPHOLOGY OF THE RENAL TUBULAR EPITHELIUM IN YOUNG, HEALTHY HUMANS

By

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Investigations of the ultrastructure of renal glomeruli showed some differences between man and laboratory animals (*Bergstrand & Bucht* 1958). Structural variations and modifications that would have been labelled pathological in animals were observed in young adult humans lacking clinical signs of renal disease. These findings indicate that anatomical studies of laboratory animals cannot be used as a reliable basis for the evaluation of pathologic glomerular changes in man.

Preliminary work showed that this conclusion is also valid for the tubular epithelium of the kidney (*Bergstrand & Ericsson* 1960). The ultrastructure of renal epithelial cells in various laboratory animals has been the subject of extensive investigations (*Dalton et al* 1950, *Pease & Baker* 1950, *Sjostrand & Rhodin* 1953, *Rhodin* 1954, 1956, 1958, 1962, 1963, *Pease* 1955, 1956, *Bargmann et al* 1955, *Clark* 1957, *Ruska et al* 1957, *Sakaguchi & Suzuki* 1958, *Sampaio et al* 1958, *Suzuki* 1958, *Novikoff* 1959, *Stadal Pour* 1959, *Miller* 1960, 1961, *Stone et al* 1961, *Trump* 1961, *Trump & Janigan* 1962, *Ericsson* 1964, *Ericsson & Trump* 1964, 1965, *Ericsson et al* 1965). The present paper reports on results of a survey study which was performed in order to obtain information for further work in human pathology. An account is given of the fine structure of the renal tubules and collecting ducts and it is emphasized that structural modifications of the tubular cells that by

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Th ... Mrs Margare ... and Mrs *Marlis Aslund* and the help during the preparation of the typescript by Mrs *Britt Marie Högberg* and Miss *Adoree Förare*

hand has less frequently been used for this purpose In the case here reported strong reasons could be adduced for the presence of a primary heart tumour proceeding from the heart wall in the light of the coronary angiography In those cases in which the presence of a primary heart tumour may be suspected one should therefore utilize the diagnostic possibilities offered by coronary angiography For the rest the histological picture in this case of a primary fibrous hamartoma of the heart appears to be the first case described in which the tumour has proceeded from the right atrium and ventricle

SUMMARY

Clinic diagnosis and pathology of a case of primary fibrous hamartoma of the heart are described

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BB
TL
BB
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N

some investigators have been interpreted as pathological may indeed occur under apparently normal conditions It appears that some of these modifications represent artifacts produced during the preparation procedures The importance of properly evaluating such appearances in studies of patients with renal disease is stressed

MATERIALS AND METHODS

Percutaneous renal biopsies using the Vim Silverman needle, have been performed on 13 young adults, 7 males and 6 females, ranging between 15 and 30 years of age Six of these persons were examined at the Istituto di Pathologia Medica dell Universita Rome, Italy, while seven were patients from the surgical or medical wards at S t Erik's Hospital Stockholm Sweden They were treated for minor ailments unrelated to kidney disease A routine examination of renal function including endogenous creatinine or inulin clearance was performed on all these subjects No signs of renal impairment were observed in any of them at the time of examination Renal biopsy was made in the morning when the patients had fasted for a period of ~ 12 hours

Immediately after the biopsy had been performed the specimen was divided and one half was fixed in 4 per cent neutral formaldehyde or Bouin's solution these tissues were embedded in paraffin and utilized for light microscopic studies of sections stained with haematoxylin & eosin, van Gieson, or PAS The remainder was fixed in 1 per cent buffered osmium tetroxide (pH 7 4) for a period of 1½–2 hours at 0–4° C (*Palade* 1952) *These specimens were further divided into small* cubes with a side of ~ 1mm, they were dehydrated in a graded series of ethanol solution starting with 30 per cent and embedded in a mixture of methyl- and butyl

were cut at 0 5 1 0 μ on a Sartorius microtome
The sections were studied in a phase contrast
and proper trimming of the blocks ultrathin sections for electron microscopy were cut using a Leitz or a Sjöstrand microtome both equipped with glass knives A second series of 0 5–1 0 μ thick sections for phase contrast *microscopy was then cut from the blocks and further sections prepared* for electron microscopy

OBSERVATIONS

1 *Light Microscopy*

Most of the proximal convoluted tubules fixed in formaldehyde and embedded in paraffin had wide, patent lumens that were more or less completely filled with a granular "debris" (Fig 1) In some tubules

---

Figs 1 2

Fig 1 Light microscopic picture of formaldehyde fixed renal cortex showing several cross sectioned proximal convoluted tubules (PT) The brush borders (BB) are usually preserved in these tubules Most of them have patent lumens containing finely granular debris (De) including many nuclei (unattached arrows) The distal convoluted tubules (DT) are characterized by lack of brush border and abundance of nuclei which usually are located close to the lumen Glomerulus (Glo) Haematoxylin & eosin stain × 700

Fig 2 Low magnification electron micrograph of part of a cross sectioned proximal convoluted tubule (PT) with patent lumen (TL) The lumen contains parts of cytoplasm which apparently represent protrusions (Pr) from the tubular ... The organization of the apical cell membrane in a brush border (BB) ... formed (between unattached ... N) Nucleolus (Nuc), Part of ... 3 450


BB
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BB
TC
DT
BB
1
N
TL
2


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AV
BB
D
ES1
De2
AV
AV
AV
ES2
BB
ES3
N
3

there was, however, no clearly visible lumen due to collapse In the distal convoluted tubules, granular or structureless material was often observed in the lumens, on the other hand, the thin limbs of Henle's loops and the collecting ducts usually appeared empty Similar observations were made on the tissues fixed in Bouin's solution and on the osmium-fixed tissues studied in the phase contrast microscope

2 *Electron Microscopy*

The appearance of the various parts of the nephron from the proximal convoluted tubules to the collecting ducts is illustrated in Figs 2 18 The nomenclature used to designate cytoplasmic organelles and structures is that suggested by *Ericsson & Trump* (1964, 1965) (summarized by *Ericsson* 1964) for corresponding images in the tubular cells of rat kidney The main features of this nomenclature will be evident from the legends for the figures

In most instances the different parts of the nephron could be easily identified through comparisons with the light microscopic findings, supplemented by studies in the phase contrast microscope Further aid in pinpointing the type of tubule in the thin sections was obtained from the similarity of most parts of the nephron with that in the rat kidney Proximal tubules located in the vicinity of a glomerulus were regarded as constituting part of the convoluted section The 'pars recta could not be identified with certainty, tubules with typical brush border that were not located close to a glomerulus were therefore referred to as just "proximal tubules' Difficulties were further encountered in properly identifying the thin (descending) limbs of Henle's loops, as discussed in the legend for Fig 8

a *Proximal tubules* (Figs 2–7) Electron microscopy showed that the luminal contents of the proximal convoluted tubules observed in the light and phase contrast microscope consisted of cellular "debris" that in some areas seemed to be in close connection or continuous with the epithelial cell cytoplasm (Figs 2 and 3) The debris' varied from isolated organelles such as mitochondria, to large pieces of cytoplasm limited by a single membrane and containing cell constituents (Fig 3) The appearance of the luminal part of proximal tubular cells is

Fig 3

Part of a proximal convoluted tubule with patent lumen (TL) which is filled with cellular organelles and areas of cytoplasm Many of these cytoplasm [illegible] clearly limited by [illegible] (Dep w[illegible] cellular [illegible] with the [illegible] at the [illegible] the way [illegible] (B [illegible] by unattached arrows in ES₂) and are apparently created through separations of the lateral cell membranes There are many apical vacuoles (AV) in the luminal parts of the cells Nucleus (N) × 8 750

shown in Figs 2–4 and 7 In some areas the apical cell membrane is smooth and apparently attenuated with disappearance of the microvilli In such areas the cytoplasm is usually bulging into the tubular lumen forming protrusions of variable size Protrusions and cellular "debris" were observed in most cross sections of the proximal tubule in all of our subjects The definitive impression was gained from observations of a large number of such tubules cut at different angles and in some instances studied in serial sections that many parts of cytoplasm which were free in the lumen and constituted the "debris" represented pinched off protrusions

The brush border is created through extensive plication of the apical cell membrane which thus forms the microvilli Tubular invaginations of the apical cell membrane ("apical tubular invaginations", Fig 7) penetrated into the cytoplasm for variable distances Some of these were dilated, notably at their bases, having the appearance of small vesicles (apical vesicles) in transverse sections The apical cytoplasm also contained apical vacuoles (larger than apical vesicles) sometimes connected with apical dense tubules (inset in Fig 7) The latter are believed to represent collapsed tubular invaginations of the apical cell membrane

In the luminal part of the cells a few, small mitochondria were observed In the intermediate and basilar parts of the cells the number and general appearance of the mitochondria (Figs 5 and 6) was similar to that seen in animals Thus, the mitochondria were long and slender and usually oriented perpendicularly to the basement membrane Mitochondrial matrix granules (Figs 5 and 6) were frequently observed

Cytosomes (Fig 6, insets) were rarely encountered, they were much less numerous than the "big granules" and "vacuolated bodies" in the proximal tubules of mouse (*Rhodin* 1954, 1958, 1961, *Miller* 1960, 1961) and the cytosomes of rat kidney (*Trump* 1961, *Trump & Janigan* 1962, *Ericsson & Trump* 1964, 1965) They were limited by a single membrane and often enclosed irregular patches of dense material as well as

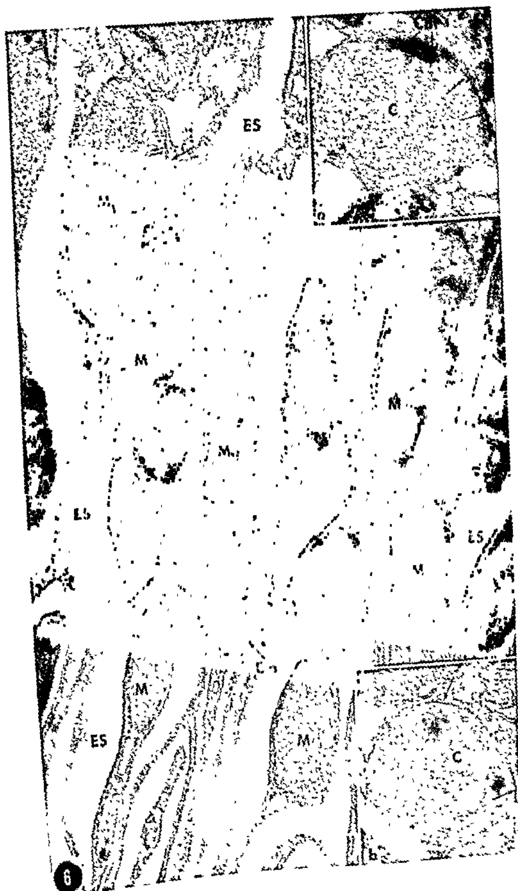
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Figs 4 5

Fig 4 Apical cytoplasm of proximal convoluted tubule forming two protrusions (Pr_1 and Pr_2) bulging into the patent tubular lumen (TL) An area of cytoplasm which apears to have been extruded into the lumen is indicated by De Two small mitochondria (M) and an apical vacuole (AV_1) are *present in the* large protrusion (Pr_1) which mainly contains granular material the small protrusion (Pr_2) contains 2 mitochondria (M) Apical vacuole (AV) Brush border (BB), Nucleus (N) × 12 000

Fig 5 Basilar cytoplasm of proximal tubular cell showing irregularly dilated extracellular spaces (ES) between the infoldings of the basilar cell membrane (BI) Transversely cut collagen fibres (Col) outside the basement membrane (BM) are closely associated with the latter No collagen is present however in the substance of the basement membrane proper which is composed of a finely granular homogenous substance Mitochondria (M) Mitochondrial matrix granules (Mg) × 60 000


BB
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AV
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De
bh
Pr
N
4
ES
ES
M
M
M
ES
BM
Br
5


ES
C
a
M
M
M
M
LS
LS
M
C
M
ES
M
C
b
6

membrane fragments (see Fig 6) So far, clearly identifiable micro bodies (*Rhodin* 1954, *Ericsson* 1964) have not been encountered

The basilar infoldings of the cell membrane (Figs 3 and 5) penetrated deep into the cell, sometimes to more than half of its height The compartments thus formed mainly contained mitochondria The extracellular spaces between the infolded membranes were often very wide forming large extracellular compartments (Figs 3, 5, and 6) Scattered ribosomes were present in the ground cytoplasm The endoplasmic reticulum was not very extensive and consisted of flattened sacs of both smooth and rough surfaced variety The basement membrane (Fig 5) showed no visible substructure The collagen fibres of the interstitial tissue were prominent but did not seem to be firmly associated with the basement membrane proper (Fig 5)

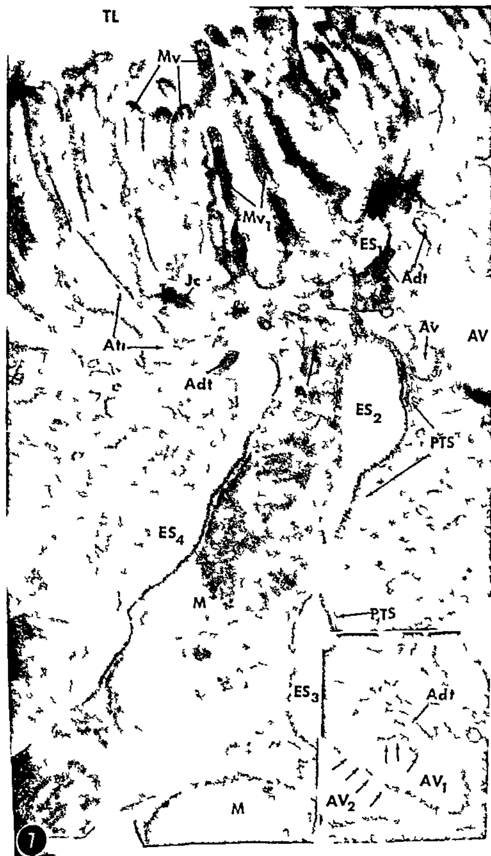
b *Loops of Henle* (Figs 8–10) Concerning the identification of the different parts of the loops of Henle see legend for Fig 8 Cross sections believed to represent one thin, descending loop and one thick, ascending loop are shown in this figure The lumens of these contained occasional small areas of cellular debris and were almost invariable patent

The cellular lining of the thin limb was attenuated (Figs 8 10) and the nuclei of the cells appeared flattened The basement membranes were thick (up to $\sim 1.5\ \mu$) and the cell surfaces smooth with occasional small villous protrusions (Fig 9) The lateral cell membranes were slightly tortuous and closely applied The basilar cell membrane did not exhibit infoldings of the type encountered in the proximal tubular cells Occasionally cytosome like bodies were present in the cytoplasm (Figs 8 and 9) which in addition contained infrequent, small mitochondria

In the thick limbs of Henle (Figs 8 and 10) the cells contained numerous elongated mitochondria The nuclei were large with smooth rounded contours There were more numerous and larger villous protrusions of the luminal cell membrane than in the thin limb Basilar infoldings of the cells membrane were numerous and extended deep into the cytoplasm The infolded basilar cell membranes were closely

Fig 6

Intermediate and basilar part of proximal tubular cell showing pronounced widening of the extracellular spaces (ES) formed between the infoldings of the ba[illegible] cell membranes (Cm) The [illegible] mitochondria (M M_1) M [illegible] [illegible] *Insel* [illegible] [illegible] ontains dense *Insel* [illegible] with [illegible] in a matrix [illegible] in *inset a* The single limiting membrane is in [illegible] unattached arrows × 60 000 *Insel a* × 52 800 *Insel b* × 84 000



TL
Mv
Mv_1
ES_1
Adt
Jc
Av
AV
Att
Adt
Av
ES_2
PTS
ES_4
M
PTS
ES_3
Adt
AV_1
AV_2
M
7

applied and widened extracellular spaces were not observed Cytosomes were rarely encountered

c *Distal convoluted tubules* (Figs 11–13) The epithelial cells (Fig 11) were approximately of the same height as those of the proximal convolutions The apical cell membrane formed short villous protrusions (Figs 11 and 13) that were numerous in some areas, sparse in others There was usually a wide lumen in which sometimes cellular "debris", mostly consisting of mitochondria, was found Large parts of cytoplasm or protrusions connected with the epithelial cells similar to those seen in the proximal tubules (see Figs 2–4) were not observed The mitochondria were long and slender and contained numerous cristae (Fig 13) The orientation of the long axis of the mitochondria was almost invariably perpendicular to the basement membrane The basilar infoldings of the cell membranes were deep but there were no distensions of the extracellular compartments as in the proximal tubular cells In the luminal parts there were many vesicles (Fig 13) Connections between them and the cell surface have not been observed The ribosomes were sometimes numerous and were usually unattached to the endoplasmic reticulum Dense granules with a size and appearance similar to that of particulate glycogen were sometimes seen in the ground cytoplasm (Fig 13) Large, dense inclusion bodies were frequently observed in the cytoplasm (Fig 11) Bodies with the appearance of lipid droplets and lipofuscin granules were also noted (Figs 11 and 12) The basement membrane was thinner than in the proximal convolutions and sometimes appeared to be split into lamellae

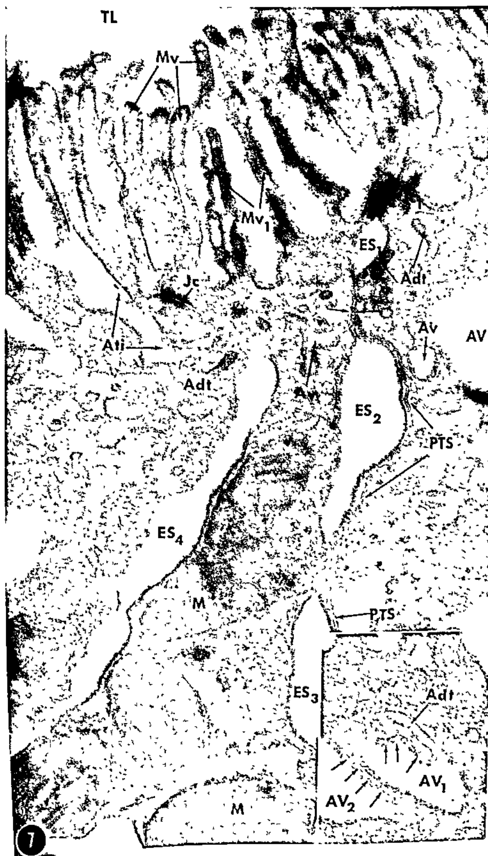
d *Collecting ducts* (Figs 14–18) The cells in the proximal parts of the collecting ducts were of two kinds smooth surfaced "light cells" with few organelles and pale ground cytoplasm, and "dark cells" containing numerous mitochondria and ribosomes (Fig 17) The latter cells were usually interspersed singly among the "light cells" which were fare more numerous and appeared with increasing frequency in

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Fig 7

Luminal part of pr ximal convoluted tubule The brush border ... usually tightly packed mi ... apart (Mv_1) Below a ju... cells are separated to f... (FS_1 FS_2 and FS_3) A... [illegible] ... FS_1 and FS_2 are indica... contains apical dense ... (AV) At least some ap... ...acuole represent cross sectioned tubular invaginations of the apical cell membrane (Ati) Note that the so-called paramembranous tubular system (PTS) closely follows the outline of the lateral cell membrane ... Mitochondria (M) renal tubular lumen (TL)

Inset An apical vacuole (AV_1) is connected with an apical dense tubule (Adt) Note that the luminal side of the wall of this and an adjacent apical vacuole (AV_2) is coated with amorphous or finely flocculent material having the same texture as the dense substance in the core of the apical dense tubule (Adt) × 44 800 Inset × 60 000


TL
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Mv1
T
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Adt
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Ati
Av
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Adt
Av
ES2
PTS
ES4
M
PTS
ES3
Adt
AV1
AV2
M
7

the more distal parts (compare Fig 14 with Fig 17) Frequent infoldings of the basilar cells membrane were noted in the cortical collecting ducts These infoldings were shallow and usually did not form compartments containing mitochondria as in the proximal and distal convoluted tubules (see Fig 17, compare with Figs 6 and 12) Some cells in the collecting ducts contained a well developed Golgi apparatus (Fig 16) Tubular or vesicular profiles of the endoplasmic reticulum were rarely encountered The mitochondria were rather small, often with ovoid shape and were dispersed randomly in the cytoplasm Occasional mitochondria presented more irregular contours (Figs 15 and 17) and some contained numerous cristae sometimes showing anastomoses and angular outlines (Fig 15) The cells of the distal parts of the collecting ducts (Fig 18) were characterized by paucity of cellular organelles, pallor of ground cytoplasm, and simplification of cell membrane organization (absence of basilar infoldings and apical villous protrusions, straight lateral cell membranes)

DISCUSSION

Our biopsy specimens were obtained from two groups of people differing in dietary habits and environmental conditions Presumably they show the characteristic appearance of normal, young human kidneys

Two main conclusions may be drawn from this study (*a*) In needle biopsies of normal renal tissue treated with conventional methods there is a considerable amount of 'debris', consisting of parts of cytoplasm and various cellular organelles, in the lumens of the tubules, notably the proximal, convoluted parts unassociated with proteinuria or other signs of impaired renal functions (*b*) The epithelial cells exhibit considerable structural variation and differ in some respects from corresponding cells in laboratory animals

Consideration must be given to the possibility that some of the

Fig 8

Low magnification electron micrograph showing the appearance of the outer medulla Three types of tubular structures are seen in the picture (*a*) A small vessel probably an arteriole (Art) (*b*) A tubule lined by [illegible] [illegible] the intercellular connective tissue (CT) is much more abundant than in the cortex Boxed area is shown at higher magnification in Fig 10 × 4,500


CT
C
CT
Des
CT
Asc
G
G
CT
O

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Fig 8

Low magnification electron micrograph showing the appearance of the outer medulla Three types of tubular structures are seen in the picture (*a*) A small vessel probably an arteriole (Art) (*b*) A tubule lined by roughly cuboidal cells with abundant mitochondria (Asc) Unattached arrows [illegible] tifiable "debris" in the lumen



structural modifications described above may be artifacts produced during the preparation of the specimens For instance, the appearance of apical tubular protrusions and cellular "debris" in tubular lumens may be due to an unavoidable compression of the tissues by the biopsy needle One type of traumatic damage is readily observed with the light microscope in all our biopsy specimens It is limited to the superficial parts of the tissues and consists mainly of rupture and compression with flattening of tubules and glomeruli Since we have not had the opportunity to compare needle biopsy specimens with surgical biopsy material from normal humans we can not, however, completely exclude the possibility that the tubular modifications mentioned above may be due, at least in part, to traumatization by the biopsy needle

It is also impossible to avoid a certain loss of time between the insertion of the biopsy needle into the renal tissue and the beginning of fixation In none of our cases, however, did more than 2 3 minutes pass between the removal of the biopsy needle with the specimen and the beginning of fixation It is unlikely that the appearances observed are to be interpreted as being due to autolysis since very insignificant if any, alterations seem to occur within such a short time period (*Trump et al* 1962)

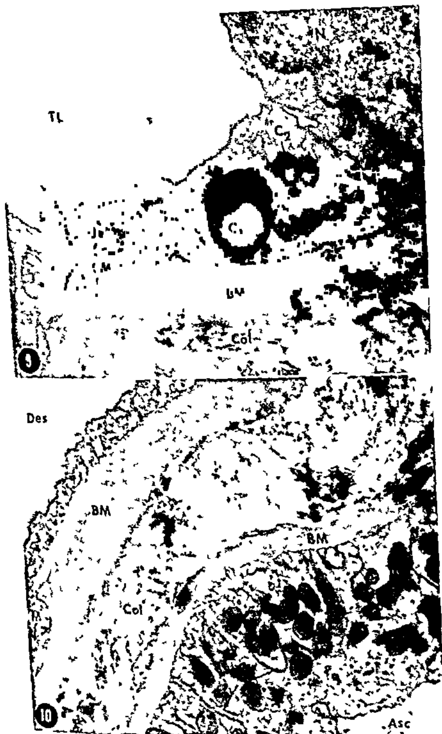
The material was collected before the introduction of embedding media such as Epon (*Luft* 1961) and Vestopal (*Ryter & Kellenberger* 1958) which are known to give a better preservation of the ultra structure than methacrylates (*Pease* 1960) The main advantage with these media seems to be that they prevent the occurrence of polymerization damage with "explosion" of the tissues so often encountered in methacrylate-embedded material It is unlikely, however, that the presence of cellular "debris" in the tubular lumens, and the formation of apical protrusions would occur during the polymerization procedure when the tissues are already fixed Furthermore, similar appearances are noted in tissues embedded in paraffin (*Longley & Burstone* 1963 see also Fig 1 of the present investigation) The evidence would therefore indicate that the presence of cellular "debris" in the lumens of the tubules is not related to the effect of the embedding medium

---

Figs 9 10

Fig 9 Part of thin limb of Henle s loop (concerning identification see legend to Fig 8) The cytoplasm contains small mitochondria (M) and two cytosome like bodies (C_1 C_2) Note the absence of infoldings of the basilar cell membrane Basement membrane (BM) Collagen (Col) Cell membrane (CM) Junctional complex (Jc) Nucleus (N) Tubular lumen (TL) small villi on the cell surface (vi) × 27 000

Fig 10 This picture shows the boxed area in Fig 8 at higher magnification The basement membrane (BM) of the thin limb of Henle s loop (Des) is much thicker than that of the thick (Asc) A suggestion of layering or lamellation is revealed in both basement membranes Infoldings of the basilar cell membrane (BI) are frequent in the cells of the thick limb Collagen in the peritubular interstitial tissue (Col) Mitochondria (M) × 14 700


TL
N
C2
C1
M
BM
Col
9
Des
BM
Col
BM
10
Asc

Studies of the appearance of the proximal convoluted tubules in rat kidney (*Pease* 1955, *Maunsbach et al* 1962, *Ericsson* 1964, *Ericsson et al* 1965) indicated that a rapid collapse of the proximal tubules with obliteration of the lumens occurred when small pieces of the renal cortex were removed and immersed in the fixative On the other hand, the lumens remained in their physiological, patent state (*Steinhausen et al* 1963) following fixation *in vivo* by dripping the fixative on the surface of the kidney (*Pease* 1955, *Maunsbach et al* 1962, *Ericsson & Trump* 1964, 1965), furthermore, the infolded basilar cell membranes were closely applied The collapse of the proximal tubules following fixation by immersion was accompanied by the presence of cellular organelles in the tubular lumens, formation of apical protrusions, and occasionally wide separations of the infolded basilar cell membranes (*Maunsbach et al* 1962, *Ericsson* 1964) These features were similar to those observed in our material of kidney biopsies, although the enlarged extracellular compartments were seen much more frequently in the human material The notion that widened extracellular spaces in the proximal tubule cells might be created as a result of tissue shrinkage known to occur during the dehydration procedure (*Bahr et al* 1957) is largely contradicted by the absence of such spaces following fixation *in vivo* If, indeed, the results from studies on experimental animals are valid also for the situation in the normal human kidney, the evidence would therefore indicate that cellular debris in the tubular lumens, apical protrusions of the proximal tubular cells, and separations of the infolded basilar cell membranes are preparation artifacts occurring during the insertion of the biopsy needle and the subsequent handling of the tissue prior to fixation Since it is not possible to perform *in vivo* fixation of the human kidney, the morphologist is obliged to study renal biopsies fixed by immersion It is therefore important to be aware of the modifications of appearance discussed above, especially when one attempts to study pathologic conditions It seems for instance that although separations of the infolded basilar cell membranes may occur under certain pathological (*Stone et al* 1961) and physiological (*Caulfield & Trump* 1962) conditions, at least in experimental animals, the proper interpretation of the significance of such appearances is extremely difficult if not impossible in kidney biopsies from humans

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Fig 11

Part of distal convoluted tubule with patent lumen (Tl) containing scattered cytoplasmic organelles some of which are probably mitochondria (unattached arrows) Mitochondria are very numerous in the cytoplasm of the tubular cells Dense cytosome like bodies (unattached arrows) are also relatively frequent Some cytoplasmic bodies (indicated by Li) probably represent lipid or lipofuscin The apical cell membrane is either smooth or forms small villous protrusions (vi) Nuclei (N)
Inset Higher magnification picture of basilar cytoplasm showing closely applied infoldings of the basilar cell membrane (BI) Basement membrane (BM) × 7 500
Inset × 51 000


11
BM

Studies of the appearance of the proximal convoluted tubules in rat kidney (*Pease* 1955, *Maunsbach et al* 1962, *Ericsson* 1964, *Ericsson et al* 1965) indicated that a rapid collapse of the proximal tubules with obliteration of the lumens occurred when small pieces of the renal cortex were removed and immersed in the fixative On the other hand, the lumens remained in their physiological, patent state (*Steinhausen et al* 1963) following fixation *in vivo* by dripping the fixative on the surface of the kidney (*Pease* 1955, *Maunsbach et al* 1962, *Ericsson & Trump* 1964, 1965), furthermore, the infolded basilar cell membranes were closely applied The collapse of the proximal tubules following fixation by immersion was accompanied by the presence of cellular organelles in the tubular lumens, formation of apical protrusions, and occasionally wide separations of the infolded basilar cell membranes (*Maunsbach et al* 1962, *Ericsson* 1964) These features were similar to those observed in our material of kidney biopsies, although the enlarged extracellular compartments were seen much more frequently in the human material The notion that widened extracellular spaces in the proximal tubule cells might be created as a result of tissue shrinkage known to occur during the dehydration procedure (*Bahr et al* 1957) is largely contradicted by the absence of such spaces following fixation *in vivo* If, indeed, the results from studies on experimental animals are valid also for the situation in the normal human kidney, the evidence would therefore indicate that cellular debris in the tubular lumens, apical protrusions of the proximal tubular cells, and separations of the infolded basilar cell membranes are preparation artifacts occurring during the insertion of the biopsy needle and the subsequent handling of the tissue prior to fixation Since it is not possible to perform *in vivo* fixation of the human kidney, the morphologist is obliged to study renal biopsies fixed by immersion It is therefore important to be aware of the modifications of appearance discussed above, especially when one attempts to study pathologic conditions It seems for instance that although separations of the infolded basilar cell membranes may occur under certain pathological (*Stone et al* 1961) and physiological (*Caulfield & Trump* 1962) conditions, at least in experimental animals, the proper interpretation of the significance of such appearances is extremely difficult if not impossible in kidney biopsies from humans

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Inset Higher magnification picture of basilar cytoplasm showing closely applied infoldings of the basilar cell membrane (BI) Basement membrane (BM) × 7 500
Inset × 51 000


N
M
B
12
TL
M
V
L
M
V
13

For instance, it becomes difficult to evaluate to what extent the expansions of the basilar extracellular spaces of proximal tubules observed in kaliopenic nephropathy in man (*Biava et al* 1963) really represent pathological alterations and not artifacts produced during the handling of the tissue

As indicated in the legend to Fig 8, certain difficulties were involved in a proper identification of the thin, descending loops of Henle It is tentatively assumed that the tubule indicated by Des in Fig 8 represents this part of the nephron This assumption is based on comparisons with the appearance in the light and phase contrast microscopes and on the fact that erythrocytes or flocculent, precipitated plasma were not present in the lumens Infoldings of the basilar cell membrane were not noted in the cells lining these tubules but were reported to occur in corresponding parts of the nephron in rabbit (*Sakaguchi & Suzuki* 1958) and mouse kidney (*Rhodin* 1958) Species differences may thus exist concerning the structure of the tubular cells in the thin loops of Henle

Due to the limitations involved in the preparation of the material (notably the fact that it was embedded before Epon and Vestopal became commercially available), fine structural details of diverse organelles could not be revealed in sufficient detail The findings did suggest, however, that there are many differences between man and experimental animals, for instance concerning structures such as microbodies, cytosomes, and the cell membrane in the proximal tubules, and the appearance of the cells of Henle's thin loop In the future it would therefore be fruitful to try to further elucidate these features using Epon- or Vestopal embedded tissues in order to further strengthen the basis for studies of the tubules during pathological conditions

SUMMARY

A survey study of the fine structure of the renal tubular epithelium in man has been performed Kidney biopsies from 13 young adult humans without clinical history or laboratory findings of renal disease were studied by light, phase contrast, and electron microscopy

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Figs 12 13

Fig 12 Basilar part of distal convoluted tubule Two large bodies are present in the cytoplasm 1 is probably a lipid droplet while 2 has the appearance of lipofuscin granules Note that the infolded basilar cell membranes (BI) are closely applied Mitochondria (M) Nucleus (N) × 24 300

Fig 13 Apical part of distal convoluted tubule The cytoplasm contains many vesicles (v) and randomly distributed small granules (unattached arrow) with a diameter of 200–300 Å which may represent glycogen granules Note the mitochondrion (M) in the tubular lumen (TL) and the small villus like projections (vi) from the cell surface Junctional complex (Jc), Mitochondria (M), multivesicular body (Mvb) × 41 600


MB
14
CM
M
Gc
GV
Gc
GV
GV
15
16

Differences in number, size, and appearance between man and experimental animals was noted concerning many cellular organelles. In the proximal convoluted tubules the fine cellular structure showed more variations in man than in animals. The cytoplasm of many proximal tubular cells formed protrusions of variable size into the lumens of the tubules which particularly in the proximal convolutions contained large amounts of cellular debris. This debris consisted of parts of cytoplasm and cellular organelles, presumably representing protrusions which had become separated from the cytoplasm of the tubular cells and been extruded into the lumen. The accumulated evidence, notably that derived from studies of experimental animals, indicates that these appearances, as well as the often encountered enlarged intercellular spaces in proximal tubular cells, probably are preparation artifacts. The importance of appreciating these modifications of structure was stressed, with special reference to the limitations they impose on morphologic studies that aim at characterizing physiological and pathological alterations in the renal tubules of man.

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Fig 14–16

Fig 14 Collecting duct in the outer medulla. The cells are cuboidal or cylindrical with rather pale matrix and contain moderate numbers of small ovoid mitochondria and occasional cytosome like bodies (C). × 5 200

Fig 15 Area of cytoplasm from collecting duct situated in the outer medulla (compare with Fig 14). Note angular configurations and branching of cristae in one of the mitochondria (M). The cytoplasmic matrix is pale. Cell membrane (CM) × 70 000

Fig 16 Golgi apparatus in the same type of cell as in Figs 14 and 15 consisting of 3 elements (*a*) large Golgi vacuoles (GV), (*b*) occasional flattened Golgi cisternae (Gc), and (*c*) small Golgi vesicles (Gv) × 57 600

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Rhod …
Rhod … Black

Figs 17 18

Fig 17 Part of collecting duct in the outer cortex showing the appearance of a "dark cell" (DC) and part of a "light cell" (LC) The "dark cell" contains numerous mitochondria and has a rather dense ground cytoplasm at higher magnification shown to contain many free ribosomes The "light cell" has a pale ground cytoplasm and contains few mitochondria Note the numerous microvilli (vi) formed by the apical cell membrane of the "dark cell" In foldings of the basilar … but … on …

Fig 18 Par… are… mer… f… (N…


DC
LC
CM
Bl
17
18

Acta path et microbiol scandinav 63 385–390 1965

Neurochemical Research Unit (Head M Schou, M D), Psychiatric Institute (Head Professor E. Stromgren, M D), Risskov Denmark and Cancer Research Institute (Head Professor J Bichel M D), The Radium Centre Aarhus Denmark

THE EFFECT OF ANAEMIA AND COBALT ON LACTIC DEHYDROGENASE ISOENZYMES IN KIDNEY TISSUES OF RABBITS AND ITS POSSIBLE RELATION TO THE ERYTHROPOIETIN PRODUCTION

By

KNUD JENSEN and EIVIND B THORLING

Received 26 ix 64

The erythropoietin production increases when the oxygen tension in blood supplying the kidneys is diminished (*Kuratowska et al* 1961) A similar effect has been found in severe anaemia and after cobalt administration (*Goldwasser et al* 1958) The increased erythropoietin production in these cases might be caused by a local hypoxia in the kidney tissues *Pfleiderer & Wachsmuth* (1961) and *Cahn et al* (1962) have suggested that the lactic dehydrogenase (LDH)-isoenzyme pattern in different tissues depends on the local oxygen tension A study of this isoenzyme pattern under different conditions might therefore reflect changes in the oxygen tension in the tissues

The purpose of the present investigation has been to find changes in the metabolism of the kidney tissue in anaemia and after cobalt administration reflecting a deficient local oxygen supply We therefore studied the LDH isoenzyme pattern in different parts of the kidneys of normal rabbits in comparison with kidneys of rabbits with severe anaemia and after cobalt administration

MATERIALS AND METHODS

White rabbits (weight 2 5–3 kg) were used for the investigation, and studied in three groups

1 *Normal rabbits*

2 *Rabbits with severe anaemia* A severe anaemia was provoked by bleeding For five days 30 ml of blood were taken daily from an ear vein Then the haematocrit value had fallen to about half the normal and after two days at that level the animals were killed

The authors wish to express their gratitude for skilled technical assistance to Mrs *Else Poulsen* Mrs *Jette Lyberth* and Miss *Else Skov*

The investigation has been supported by grants from *Carlsbergfondet Kong Christian den Tiendes Fond* and F L Smidth & Co A/S's *Jubilæumsfond* the *Danish Anti-Cancer League* and the *Danish League against Multiple Sclerosis*

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2 [illegible]
[illegible]
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3 *Rabbits challenged with cobalt* The rabbits were injected subcutaneously with 10–20 mg of cobalt (as cobalt chloride in 10 ml 0 9 per cent solution of sodium chloride) After 24 hours the animals were killed and used for the investigations.

After killing the rabbits blood was withdrawn for determination of the haematocrit value, serum for determination of erythropoietin content, serum and urine for determination of the LDH isoenzymes

Preparation of Kidney Tissue for the Examination

The rabbits were killed by an injection of 30 ml of air into the ear vein The kidneys were taken out and cut from pole to pole in slices, about 3 mm thick

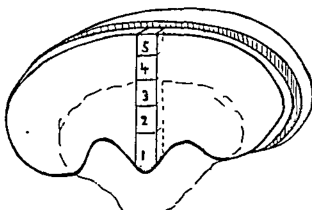


Fig 1

One slice containing parts of cortex and the pyramid including the apex was chosen From this slice samples were taken in sequence from apex to cortex (*Fig 1* Samples 1, 2, and 3 represent the medullary substance, 4 and 5 the cortical substance) The weight of each sample was about 30 mg After rinsing in saline the tissue samples were homogenized in a MSE Ultrasonic Disintegrator kept cold with ice, and then centrifugation was performed at 18000 × g at 0° C for 30 minutes in a MSE Refrigerator Centrifuge

– (LDH) isoenzymes were determined after agar gel electro
n a Vitatron electrophoresis apparatus
ained with the method of *van der Helm*
can densitometer at 595 nm On urine
itration to about 1/50 of the original
pplied by Membranfilter Gesellschaft

he Fe^{59} incorporation in erythrocytes
rk 1964)

RESULTS

The lactic dehydrogenase isoenzyme pattern changes gradually from the cortex to the apex in kidneys of normal rabbits (*Fig 2*, right) In cortex the most anodic fractions (isoenzymes 1 and 2) predominate, in the apex a strong predominance of the cathodic fractions (isoenzymes 3, 4, and 5) is found The presence of the most cathodic fraction (isoenzyme 5) in kidneys of normal rabbits has only been found in apex of the pyramid (samples 1 and 2) In rabbits with severe anaemia five isoenzymes are present in all parts of the kidney (*Fig 1*, left)

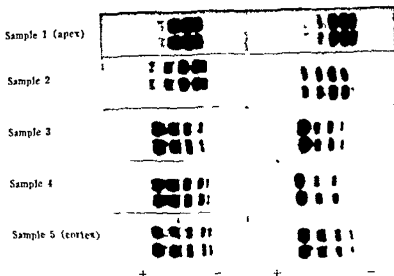


Fig 2
LDH isoenzyme pattern in kidney tissues of anaemic (left) and normal rabbits (right)

In the rabbits given cobalt the LDH-isoenzyme patterns of the samples of kidney tissue were similar to those of the anaemic rabbits (*Fig 3*)

The strongest difference between the normal, the anaemic, and the cobalt treated rabbits were found in the most superficial part of cortex and in the part of the medullary substance just beneath the cortex (*Fig 1* sample 5, and sample 3, respectively) We therefore made a special study of these parts of the kidneys

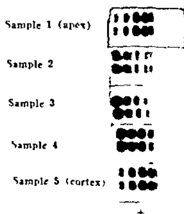


Fig 3
LDH isoenzyme pattern in kidney tissue of cobalt treated rabbit

TABLE 1

LDH-Isoenzyme 5 in the Cortical and Medullary Substances of Kidneys from Normal Rabbits, Anaemic Rabbits, and Rabbits Given Cobalt

	(Relative concentrations)		
	Number of rabbits	cortex (per cent)	Medulla (per cent)
Normal rabbits	5	0 (0 – 0 2)	0 5 (0 – 2 2)
Anaemic rabbits	7	3 9 (0 2 – 9 3)	5 4 (1 3 – 10 6)
Rabbits given cobalt	6	7 7 (2 3 16 9)	1 7 (0 – 3 6)

Table 1 shows the average relative concentration of the most cathodic isoenzyme (isoenzyme 5) Both in cortical and medullary substances of the kidneys we found a pronounced difference in the relative concentration of isoenzyme 5 when normal rabbits were compared with anaemic rabbits and with rabbits treated with cobalt

The isoenzyme patterns of serum of normal, anaemic, and cobalt treated rabbits are shown in *Fig 4*

Both in anaemic and cobalt-treated rabbits small amounts of the cathodic isoenzymes are present in serum In order to exclude the possible influence of the serum isoenzymes on the isoenzyme patterns in kidney samples, we have removed residual blood from the kidneys of three anaemic rabbits by perfusion of saline through the renal artery This procedure caused no change in the isoenzyme pattern of the kidney samples

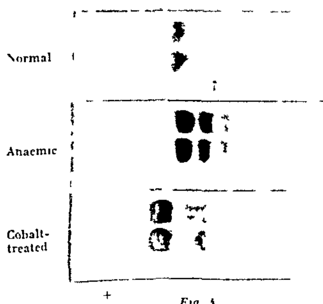


Fig 4

In urine we found in all cases only the most anodic isoenzymes (isoenzymes 1 and 2)

The erythropoietin studies of anaemic and cobalt treated animals showed the expected increase in the erythropoietin production

DISCUSSION

In the present study we found a characteristic variation in the LDH isoenzyme pattern in different parts of the normal rabbit kidney From cortex to apex of the pyramid the isoenzyme pattern changed gradually from a predominance of the anodic to a predominance of the cathodic isoenzymes Using another method *Fine et al* (1963) came to a similar conclusion According to *Pfleiderer & Wachsmuth* (1961) and *Cahn et al* (1962) an isoenzyme pattern with a predominance of the anodic isoenzymes should be characteristic for tissues with high oxygen tension while a predominance of the cathodic isoenzymes should be characteristic for tissues with low oxygen tension *Pappenheimer & Kinter* (1956) and *Kinter & Pappenheimer* (1956) supposed that the medullary substance of the kidney receives a lower oxygen supply than cortex because the red cells are separated progressively from the plasma by a process of skimming in the interlobular arteries The theory had support by *Ulfendahl* (1962) who measured the oxygen tension in different parts of the kidney The result of his investigation was that apex had a lower oxygen tension than cortex which also was the conclusion of our study

In kidneys from rabbits with severe anaemia we found a change in the isoenzyme pattern in cathodic direction when compared with kidneys of normal rabbits It seems reasonable to suggest that this change reflects a fall in the local oxygen tension caused by the anaemia

After treatment with cobalt we found changes in the isoenzyme pattern of the kidneys similar to those present in severe anaemia A conceivable explanation might be that cobalt inhibits some oxydative enzymes (*Levy et al* 1950)

The conclusion of our study is that the changes in the LDH isoenzyme pattern of the kidney tissue in severe anaemia and after cobalt administration reflect a local deficiency in the oxygen supply which might be of importance for the increased erythropoietin production

SUMMARY

The erythropoietin production increases when the oxygen tension in blood supplying the kidneys is diminished [illegible]

[illegible] tissue in anaemia and after cobalt administration reflecting a deficient local oxygen supply We therefore studied the lactic dehydrogenase (LDH) isoenzyme pattern in different parts

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LDH Isoenzyme 5 in the Cortical and Medullary Substances of Kidneys from Normal Rabbits Anaemic Rabbits and Rabbits Given Cobalt

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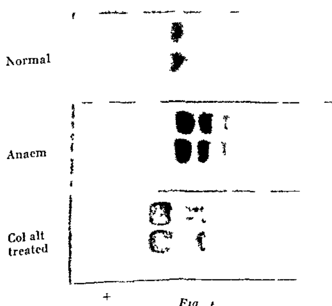


Fig 4

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The University of Bergen School of Medicine,
the Gade Institute, Department of Pathology, Bergen, Norway
(Head: Erik Waaler, M.D.)

ATHEROSCLEROSIS IN AN AUTOPSY SERIES

3. *Inter-Relationship between Atherosclerosis in the Aorta, the Coronary and the Cerebral Arteries*

By

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It is generally agreed upon that the appearance and the development of atherosclerosis depend on systemic and local factors. The former exert their influence on the entire arterial system, whereas the latter modify this influence in the various parts of the system. The result is a variation in the degree of atherosclerosis in the different arteries, and a patchy distribution of the lesions within the single artery. It seems therefore that an intimate knowledge of the inter-relationship between atherosclerosis in the various arteries should be of importance, but this knowledge is rather limited. One main reason for this lies in the many grading problems of atherosclerosis. The lack of a completely objective and sufficiently detailed grading method has prevented a direct comparison of the findings, and the usual procedure in the examination of the inter-relationship has been to compare the relation of atherosclerosis in different arteries to a common denominator, such as age or sex (see f. i. *Baker, Iannone & Kinnard* (1), *Gore & Hirst Jr.* (4), *Gore, Robertson, Hirst, Hadley & Koseki* (5), *Hirst Jr., Piyaratn & Gore* (7), *Holman & Moossy* (9), *Mathur, Patney & Kumar* (10), *Spain & Bradess* (14), *Winter, Sayre, Millikan & Barker* (16)). In this way one may achieve a general impression of the inter-relationship, but details are not obtainable.

Only a few authors have compared the findings in different arteries directly without regard to any other factors. Thus *Groom, McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (6) compared aortic and coronary atherosclerosis. In addition to the aorta and the coronary arteries, *Clagett, Rowley & Kohut* (3) included the renal arteries, and *Munck* (11) the cerebral arteries in the comparison. Furthermore, *Winter, Sayre, Millikan & Barker* (16) and *Young, Gofman, Malamud, Simon & Waters* (17) compared coronary and cerebral atherosclerosis

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of the kidneys of normal rabbits in comparison with kidneys of rabbits in severe anaemia and after cobalt administration

The LDH isoenzyme pattern was studied in samples of kidney tissue taken in sequence from cortex to apex of the pyramid In this sequence the isoenzyme pattern changes gradually from a predominance of the anodic to a predominance of the cathodic isoenzymes In anaemia and after cobalt administration a change in the isoenzyme pattern in cathodic direction was found in all parts of the kidneys This change reflects a local deficiency of the oxygen supply which might be of importance for the increased erythropoietin production

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TABLE 1

Age and Sex Distribution of the Series

Age	Number of cases		
	Males	Females	Total
15–19	3	–	3
20–29	4	5	9
30–39	17	19	36
40–49	19	17	36
50–59	37	31	68
60–69	57	48	105
70–79	49	53	102
80–89	25	24	49
Total	211	197	408
Mean Age	62.3	62.9	62.6
Age S.D.	15.8	15.8	15.8

TABLE 2

The Distribution of the Series within Specified ChPh Groups

ChPh value	Number of cases		
	Aorta	Coronary arteries	Cerebral arteries
0.00–0.19		1	7
0.20–0.39	1	6	38
0.40–0.59	9	15	93
0.60–0.79	38	13	59
0.80–0.99	25	15	38
1.00–1.19	32	23	28
1.20–1.39	33	32	28
1.40–1.59	45	26	20
1.60–1.79	53	42	18
1.80–1.99	52	59	21
2.00–2.19	48	67	15
2.20–2.39	43	49	15
2.40–2.59	15	32	10
2.60–2.79	12	16	7
2.80–2.99	1	8	5
3.00–3.19		1	4
3.20–3.39		3	
3.40–3.59	1	1	2
Total	409	409	408

RESULTS

The Total and Mean Amounts of Atherosclerosis in the Series

The sum total of all the ChPh values was 662.92 in the aortas, 732.47 in the coronary arteries and 444.72 in the cerebral arteries. This gives a ratio of 0.91 between aortic and coronary atherosclerosis, 1.49 be-

while *Reef & Isaacson* (12) compared the degree of atherosclerosis in all major arteries. The atherosclerotic process was found to follow the same general trend in the compared arteries, that is, progress in one artery was usually followed by a progress in the other ateries. There were, however, numerous individual exceptions to this rule. Other authors have also noted the frequent discrepancies in the degree of atherosclerosis in different arteries, and warned against predicting the degree of atherosclerosis in one artery from the findings in another (*Baker, Iannone & Kinnard* (1), *Reef & Isaacson* (12)). This is in obvious contrast to *Holman, McGill Jr., Strong & Geer*'s (8) statement that the aorta is a "reasonably good reflector of this change in the entire arterial tree".

In a previous paper (*Giertsen* (2)) it has been shown that the total cholesterol/phospholipoid ratio in the arterial wall—the ChPh-value—is a valid index of atherosclerosis in the aorta, the coronary and the cerebral arteries. With the help of this value a detailed examination can be made of the inter-relationship between atherosclerosis in these arteries. It should possibly be emphasized that, when this value is applied, one should not think of atherosclerosis as more or less visible changes in the arteries, but only as a measurable quantity, a mathematical symbol, which may be subjected to the ordinary mathematical operations.

MATERIAL

The material has been previously described (*Giertsen* (2)). It consists of 417 cases, 216 males and 201 females, in which the aorta, the coronary and the cerebral arteries have been examined. However, analyses of the coronary and/or cerebral arteries are lacking in 8 cases, partly for technical reasons, partly because the brain could not be examined in some cases. These 8 cases have been omitted from the series, as it would be inconvenient to operate with a different number of cases for the various arteries. The highest ChPh-value in the series was 3.59, with the exception of one coronary value of 5.66. This is such an extraordinarily high value that there is a strong reason to believe that some error has been made in the analyses. Therefore, this case has also been omitted. The age-sex distribution and the causes of death (in brackets) of the 9 omitted cases are as follows: 20–29 years: 2 males (diabetes mellitus, spontaneous pneumothorax) and 1 female (lymphosarcoma), 40–49 years: 1 male (cerebral haemorrhage), 60–69 years: 1 male (bronchial carcinoma), 70–79 years: 1 male (atherosclerotic heart disease) and 2 females (cerebral softening, subdural haematoma), 80–89 years: 1 female (atherosclerotic heart disease). The remaining 408 cases (211 males and 197 females) form the basis of this and subsequent papers. Although the new age and sex distribution are without interest in this paper, it is given for future reference in Table 1. It will be seen that the mean age (males 62.3, females 62.9, total series 62.6) and the standard deviation of the mean (15.8) are identical in the two sexes. This is purely coincidental.

...egard to the correlation be-
... cerebral arteries without
...roup the mean ChPh value
... the standard error of the
mean has also been calculated

TABLE 3

The Correlation between Corresponding Aortic and Coronary ChPh Values

ChPh group	Aorta 0 00–0 19	0 20–0 39	0 40–0 59	0 60–0 79	0 80–0 99	1 00–1 19	1 20–1 39	1 40–1 59	1 60–1 79	1 80–1 99	2 00–2 19	2 20–2 39	2 40–2 59	2 60–2 79	2 80–2 99	3 00–3 19	3 20–3 39	3 40–3 59
0 00–0 19				1														
0 20–0 39			3	3														
0 40–0 59			3	10	1			1										
0 60–0 79		1	1	4	3	2	1	1										
0 80–0 99			1	6	3	1	2	2										
1 00–1 19			1	4	4	3	2	5	3	1								
1 20–1.39				5	5	4	7	2	4	2	1			2				
1 40–1 59				1	3	4	4	7	3	2	1		1					
1 60–1 79				2	2	3	6	3	7	8	6	3		2				
1 80–1 99				2		6	4	12	9	11	11	3	1					
2 00–2 19					2	4	2	7	13	12	10	12	2	2				1
2 20–2 39						1	2	2	6	7	9	13	5	3				
2 40–2 59					1		3	3	4	7	6	4	2	1	1			
2 60–2 79					1	2			2	1	1	5	3	1				
2 80–2 99						1			1		3	1	1	1				
3 00–3 19												1						
3 20–3.39						1			1	1								
3 40–3 59												1						

Correlation coefficient r = + 0 65

TABLE 4

The Correlation between Corresponding Aortic and Cerebral ChPh Values

Cerebral arteries ChPh group	Aorta 0 00–0 19	0 20–0 39	0 40–0 59	0 60–0 79	0 80–0 99	1 00–1 19	1 20–1 39	1 40–1 59	1 60–1 79	1 80–1 99	2 00–2 19	2 20–2 39	2 40–2 59	2 60–2 79	2 80–2 99	3 00–3 19	3 20–3 39	3 40–3 59
0 00–0 19			1	4	1	1												
0 20–0 39			2	10	8	5	3	5	10	3				1				
0 40–0 59		1	4	17	8	13	13	8	12	11	4	1	1					
0 60–0 79			2	6	4	6	7	7	10	6	4	5	1	1				
0 80–0 99				1	2	2	4	11	1	5	6	5		1				
1 00–1 19						1	3	3	6	4	5	4	1	1				
1.20–1 39					2	1	1	2	4	4	4	4	4	2				
1 40–1 59						2		4	4	6	2	1						1
1 60–1 79						1	2	2	6	2	2	3						
1 80–1 99								1	4	2	7	4		3				
2 00–2 19									3	2	4	2	3	1				
2.20–2 39								1		4	3	4	2	1				
2 40–2 59								1	2	1	1	3	1	1				
2 60–2 79										2	2	1	2					
2 80–2 99											3	2						
3 00–3 19												3			1			
3 20–3 39																		
3 40–3 59											1	1						

Correlation coefficient r = + 0.38

tween aortic and cerebral atherosclerosis, and 1 65 between coronary and cerebral atherosclerosis In other words, in the total series the aortas showed about 10 per cent less atherosclerosis than the coronary arteries, and about 50 per cent more than the cerebral arteries, whereas the coronary arteries showed 65 per cent more than the cerebral arteries The mean amount of atherosclerosis was 1 62 in the aortas, 1 79 in the coronary arteries, and 1 09 in the cerebral arteries

The Distribution of the Series within Specified ChPh-Groups, i e Groups of a Specified Amount of Atherosclerosis

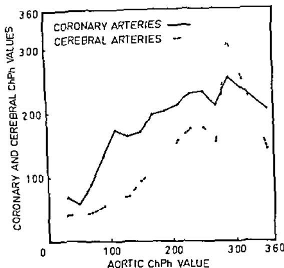
It appears from Table 2 that no aortas, only 1 coronary artery, and as many as 7 cerebral arteries were found in the first ChPh-group (0 00–0 19) The actual lowest values found were 0 38 in the aortas, 0 18 in the coronary arteries, and 0 09 in the cerebral arteries It futher appears that both the aortas and the coronary arteries are concentrated in the ChPh- interval 0 40 to 2 79, whereas more than half of the cerebral arteries show values lower than 1 00 No artery showed a ChPh-value higher than 3 59 The actual highest values were 3 55 in the aortas, 3 49 in the coronary arteries, and 3 59 in the cerebral arteries

The Correlation between the ChPh-Values in the three Arteries

Tables 3, 4 and 5 show the correlation between the ChPh-values in the aorta and the coronary arteries, aorta and cerebral arteries, and coronary and cerebral arteries The correlation coefficients are + 0 65, + 0 58, and + 0 50, respectively Thus, there is a fairly high degree of positive correlation between atherosclerosis in the three arteries, denoting that an increase in the amount of atherosclerosis in one artery usually is followed by an increase in the other arteries It is obvious from the tables, however, that the individual variations are great Thus, individuals with identical aortic values may show widely differing coronary values and vice versa (Table 3)

Furthermore, the aortic-cerebral distribution is somewhat uneven (Table 4) as it is "drawn up" towards the right upper corner of the table It is apparent that low aortic values are always combined with low cerebral values, whereas high aortic values may be combined with widely differing cerebral values On the other hand, low cerebral values may be combined with widely differing aortic values, whereas high cerebral values always are combined with high aortic values

A similar trend is apparent in the coronary-cerebral correlation (Table 5) Thus, low coronary values are always combined with low cerebral values, but the higher coronary values may be combined with widely differing cerebral values On the other hand, low cerebral values may be combined with widely differing coronary values, whereas high cerebral values practically always are combined with high coronary values



F g 1
The mean coronary and cerebral ChPh values at specified aortic ChPh groups

TABLE 6
The Mean Coronary and Cerebral ChPh Values at Specified Aort c ChPh Groups

Aorta ChPh group	ChPh value			
	Coronary arter es		Cerebral arteries	
	Mean	*S E*	*Mean*	*S E*
0 00–0 19				
0 20–0 39	0 68		0 40	
0 40–0 59	0 58	0 08	0 44	0 06
0 60–0 79	0 89	0 07	0 45	0 03
0 80–0 99	1 32	0 11	0 55	0 06
1 00–1 19	1 74	0 10	0 67	0 06
1 20–1 39	1 63	0 08	0 70	0 06
1 40–1 59	*1 70*	*0 07*	*0 94*	*0 08*
1 60–1 79	1 97	0 07	1 14	0 08
1 80 1 99	2 03	0 05	1 19	0 10
2 00 2 19	2 12	0 05	1 57	0 11
2 20 2 39	2 29	0 05	1 75	0 13
2 40 2 59	2 32	0 09	1 76	0 13
2 60–2 79	2 09	0 14	1 53	0 20
2 80–2 99	2 53		3 10	
3 00 3 19				
3 20 3.39				
3 40 3 59	2 04		1 43	

first well represented coronary group (0.20 0 39) the aortic value is somewhat higher than and the cerebral value very close to the coronary value 0.59 and 0 23 respectively From these initial values both values rise with increasing coronary value, although at a slower rate

TABLE 5

The Correlation between Corresponding Coronary and Cerebral ChPh Values

	ChPh-group	Coronary arteries																	
		0.00–0.19	0.20–0.39	0.40–0.59	0.60–0.79	0.80–0.99	1.00–1.19	1.20–1.39	1.40–1.59	1.60–1.79	1.80–1.99	2.00–2.19	2.20–2.39	2.40–2.59	2.60–2.79	2.80–2.99	3.00–3.19	3.20–3.39	3.40–3.59
Cerebral arteries	0.00–0.19	1	3	1			2												
	0.20–0.39		2	6	4	3	4	7	1	3	5	3							
	0.40–0.59		1	4	8	6	8	11	7	12	14	11	3	4	2	1		1	
	0.60–0.79			3	1	5	2	6	5	13	5	8	3	1	6	1			
	0.80–0.99					1	3	3	4	4	9	3	5	3	2	1			
	1.00–1.19							1	3	2	5	4	8	2	1	1		1	
	1.20–1.39			1			2	1	3		6	4	5	6					
	1.40–1.59							1	3	2	2	7	3	1	1				
	1.60–1.79										6	8	2	1	1				
	1.80–1.99						1	1		3	1	5	7	3					
	2.00–2.19									1	3	5	3	1	1			1	
	2.20–2.39							1			1	4	4	4		1			
	2.40–2.59						1					3	1	3		2			
	2.60–2.79									2		1	2		2				
	2.80–2.99										2		1	1		1			
	3.00–3.19												1	2					1
	3.20–3.39																		
	3.40–3.59											1					1		

Correlation coefficient r = + 0.50

The Mean ChPh-Values in two Arteries at Specified ChPh-Groups in the Third

The correlation coefficients found indicate that the amount of atherosclerosis usually increases concurrently in the three arteries The average increase may be examined through a comparison of the mean values in two arteries which correspond to a specified value in the third

Table 6 and Fig 1 show the relation between specified aortic values and the corresponding mean coronary and cerebral values Both the latter values increase with the former, but the course of the curves is not identical In the first well-represented aortic ChPh-group (0.40 0.59), the coronary and cerebral values are very close to the aortic value, 0.58 and 0.44, respectively From these initial values, the mean coronary value increases faster than, and the cerebral value more slowly than, the aortic value until this has reached 1.20 Subsequently, the increase in the coronary value is somewhat slower than, and that of the cerebral value almost parallel to, that of the aortic value until this has reached about 2.50 Thereafter, both the coronary and the cerebral values become somewhat irregular with a further increase of the aortic value

Table 7 and Fig 2 show the relation between specified coronary values and the corresponding mean aortic and cerebral values In the

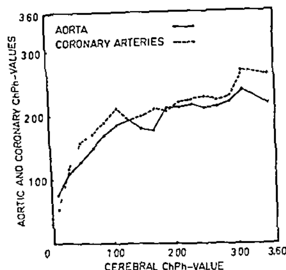


Fig 3
The mean aortic and coronary ChPh values at specified cerebral ChPh groups

TABLE 8

The Mean Aortic and Coronary ChPh Values at Specified Cerebral ChPh Groups

Cere[b]ral arteries ChP[h] group	ChPh value			
	Aorta		Coronary arteries	
	Mean	S.E.	Mean	S.E.
0.00–0.19	0.76	0.06	0.54	0.14
0.20–0.39	1.10	0.08	1.17	0.09
0.40–0.59	1.28	0.05	1.56	0.06
0.60–0.79	1.50	0.07	1.71	0.08
0.80–0.99	1.70	0.08	1.89	0.08
1.00–1.19	1.85	0.08	2.11	0.09
1.20–1.39	1.94	0.05	1.94	0.10
1.40–1.59	1.79	0.12	1.99	0.07
1.60–1.79	1.77	0.08	2.10	0.06
1.80–1.99	2.12	0.08	2.07	0.08
2.00–2.19	2.13	0.07	2.20	0.11
2.20–2.39	2.16	0.09	2.24	0.10
2.40–2.59	2.11	0.11	2.28	0.20
2.60–2.79	2.13	0.08	2.22	0.15
2.80–2.99	2.20		2.29	
3.00–3.19	2.38		2.68	
3.20–3.39	–		–	
3.40–3.59	2.17		2.62	

Table 8 and Fig 3 show the relation between specified cerebral ChPh values and the corresponding mean aortic and coronary values. It appears that in the 0.00–0.19 cerebral ChPh group the aortic value is slightly higher than the coronary value, and both are considerably

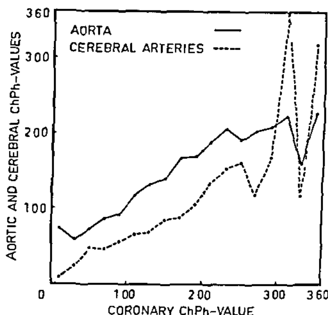


Fig 2

The mean aortic and cerebral ChPh-values at specified coronary ChPh groups

TABLE 7

The Mean Aortic and Cerebral ChPh-Values at Specified Coronary ChPh Groups

Coronary arteries ChPh group	ChPh value			
	Aorta		Cerebral arteries	
	Mean	S E	Mean	S E
0 00–0 19	0 75		0 09	
0 20–0 39	0 59	0 05	0 23	0 05
0 40–0 59	0 72	0 06	0 48	0 07
0 60–0 79	0 87	0 08	0 46	0 03
0 80–0 99	0 93	0 07	0 57	0 05
1 00–1 19	1 18	0 09	0 66	0 10
1 20–1 39	1 33	0 09	0 69	0 08
1 40–1 59	1 41	0 08	0 86	0 07
1 60–1 79	1 68	0 07	0 89	0 09
1 80–1 99	1 70	0 05	1 06	0 08
2 00–2 19	1 89	0 05	1 35	0 09
2 20–2 39	2 07	0 05	1 55	0 09
2 40–2 59	1 92	0 08	1 62	0 10
2 60–2 79	2 04	0 12	1 19	0 19
2 80–2 99	2 09	0 24	1 69	0 35
3 00–3 19	2 22		3 59	
3 20–3 39	1 60		1 19	
3 40–3 59	2 27		3 19	

Consequently, the aortic values soon become lower than the coronary values The cerebral values are consistently lower than the coronary values Above a coronary value of about 2 40 both the aortic and the cerebral values are somewhat irregular

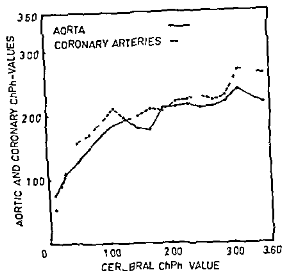


Fig 3

The mean aortic and coronary ChPh values at specified cerebral ChPh groups

TABLE 8

The Mean Aortic and Coronary ChPh Values at Specified Cerebral ChPh Groups

Cerebral arteries ChPh group	ChPh value			
	Aorta		Coronary arteries	
	Mean	S E	Mean	S E
0.00–0.19	0.76	0.06	0.54	0.14
0.20–0.39	1.10	0.08	1.17	0.09
0.40–0.59	1.28	0.05	1.36	0.06
0.60–0.79	1.50	0.07	1.71	0.08
0.80–0.99	1.70	0.08	1.89	0.08
1.00–1.19	1.85	0.08	2.11	0.09
1.20–1.39	1.94	0.05	1.94	0.10
1.40–1.59	1.79	0.12	1.99	0.07
1.60–1.79	1.77	0.08	2.10	0.06
1.80–1.99	2.12	0.08	2.07	0.08
2.00–2.19	2.13	0.07	2.20	0.11
2.20–2.39	2.16	0.09	2.24	0.10
2.40–2.59	2.11	0.11	2.28	0.20
2.60–2.79	2.13	0.08	2.22	0.15
2.80–2.99	2.20		2.29	
3.00–3.19	2.38		2.68	
3.20–3.39				
3.40–3.59	2.17		2.62	

Table 8 and Fig 3 show the relation between specified cerebral ChPh values and the corresponding mean aortic and coronary values It appears that in the 0.00–0.19 cerebral ChPh group the aortic value is slightly higher than the coronary value, and both are considerably

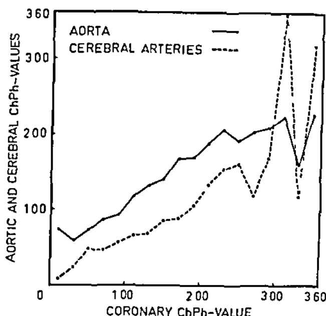


Fig 2

The mean aortic and cerebral ChPh values at specified coronary ChPh groups

TABLE 7

The Mean Aortic and Cerebral ChPh-Values at Specified Coronary ChPh Groups

Coronary arteries ChPh group	ChPh value			
	Aorta		Cerebral arteries	
	Mean	S.E.	Mean	S.E.
0.00–0.19	0.75		0.09	
0.20–0.39	0.59	0.05	0.23	0.05
0.40–0.59	0.72	0.06	0.48	0.07
0.60–0.79	0.87	0.08	0.46	0.03
0.80–0.99	0.93	0.07	0.57	0.05
1.00–1.19	1.18	0.09	0.66	0.10
1.20–1.39	1.33	0.09	0.69	0.08
1.40–1.59	1.41	0.08	0.86	0.07
1.60–1.79	1.68	0.07	0.89	0.09
1.80–1.99	1.70	0.05	1.06	0.08
2.00–2.19	1.89	0.05	1.35	0.09
2.20–2.39	2.07	0.05	1.55	0.09
2.40–2.59	1.92	0.08	1.62	0.10
2.60–2.79	2.04	0.12	1.19	0.19
2.80–2.99	2.09	0.24	1.69	0.35
3.00–3.19	2.22		3.59	
3.20–3.39	1.60		1.19	
3.40–3.59	2.27		3.19	

Consequently, the aortic values soon become lower than the coronary values. The cerebral values are consistently lower than the coronary values. Above a coronary value of about 2.40 both the aortic and the cerebral values are somewhat irregular.

atherosclerosis Likewise, the cerebral arteries may look normal or show only slight visible changes at greatly advanced coronary atherosclerosis

Our findings are generally in agreement with those of other authors Thus, *Groom, McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (6) noted a similar relationship between aortic and coronary atherosclerosis, especially in Haitian Negroes, less prominent in American Negroes *Glagov, Rowley & Kohut* (3) found that 60 out of 176 cases showed less, and 116 more, atherosclerosis in the coronary arteries than in the thoracic aorta This was called "relative sparing" and "relative involvement" of the coronary arteries as compared with the aorta As the abdominal aorta showed more atherosclerosis than the thoracic aorta, the number of coronary arteries with "relative sparing" would probably have been higher if the coronary arteries had been compared with the entire aorta

Winter, Sayre, Millikan & Barker (16) found that extreme cases might show advanced coronary atherosclerosis, but only minimal cerebral atherosclerosis, and vice versa In *Young, Gofman, Malamud, Simon & Waters'* (17) series did 2 cases display more cerebral than coronary atherosclerosis, and 8 cases more coronary than cerebral atherosclerosis In the remaining 27 cases there was agreement between the gradings in the coronary and the cerebral arteries Our findings agree with this Table 5 shows that severe coronary atherosclerosis may be combined with widely varying amounts of cerebral atherosclerosis Furthermore, although severe cerebral atherosclerosis usually is combined with severe coronary atherosclerosis, occasional cases display more cerebral than coronary atherosclerosis

As atherosclerosis is considered to be the same process in all arteries, and as systemic factors are believed to play an important aetiological rôle, a close correlation between the degree of atherosclerosis in the different arteries, as found in this series, might be expected Our coefficient for the correlation between coronary and cerebral atherosclerosis (+ 0 50) agrees very well with that (+ 0 59) found by *Young, Gofman, Malamud, Simon & Waters* (17) Comparable data of the inter-
[illegible]

[illegible] that the close correlation found only indicates that the progress of atherosclerosis in one artery, on an average, follows that in another artery The great individual variations clearly show that one cannot predict the amount of atherosclerosis in one artery from the findings in another artery alone, in a single individual Furthermore it seems possible that the results of an examination of various factors influence on the pathogenesis of atherosclerosis to some extent may depend on which artery is examined It seems also reasonable to contend that the individual variations in the interrelationship may be due to the effect of local factors in the arterial

higher than the cerebral value Already in the next group is the coronary value higher than the aortic value, and as both increase, this relation is maintained with increasing cerebral value

DISCUSSION

In this series the total amount of atherosclerosis was slightly higher in the coronary arteries than in the aortas This agrees with *Munck's* (11) findings, but these were based on a highly selected material, *viz* 396 individuals, all of whom had died from complications of coronary atherosclerosis Most authors agree that atherosclerosis is most severe in the aorta (*Gore & Hirst Jr* (4), *Groom, McKee, Webb, Grant, Pean, Hudicourt & Dallemand* (6), *Hirst, Piyaratn & Gore* (7), *Holman & Moossy* (9), *Roberts Jr, Moses & Wilkins* (13)) This discrepancy may be due to the different grading systems employed The cited authors have used a macro- or microscopical grading system, in which normal-looking arteries of course do not carry any weight However, it has been stressed previously (*Giertsen* (2)) that a grossly normal artery not necessarily has to be chemically normal ChPh-intervals were namely found, within which the arteries might or might not display slight visible changes It was contented that local factors in the arterial wall probably decide when and where an atherosclerotic lesion shall appear, and that two arteries with identical ChPh-values had reached the same stage in the atherosclerotic process, even if one looked normal and the other showed slight changes Thus, when the ChPh-value is employed as an index of atherosclerosis, normal-looking arteries may also carry weight and add to the sum total of atherosclerosis The *ChPh*-intervals mentioned were 0 38–0 51 in the aorta, 0 37–1 00 in the coronary arteries, and 0 27–1 24 in the cerebral arteries Below these intervals the arteries were invariably normal-looking, above them they always displayed visible changes The interval is narrowest *in the aorta*, indicating that this artery can bear only a slight increase in the ChPh-value *before visible changes appear In other words, on an average, the* atherosclerotic lesions appear earliest, that is at the lowest ChPh-value, in the aorta, *later in the coronary arteries and latest in the cerebral* arteries It should be stressed that the difference between the amount of aortic and coronary atherosclerosis is very small, and a change in the composition of the series might easily produce different results

These *ChPh-intervals* are of interest from another point of view as well It appears from Table 3 that aortic values up to 1 59 may be combined with coronary values lower than 1 00 from Table 4 that aortic values up to 2 79 may be combined with cerebral values lower than 1 24, and from Table 5 that even the highest coronary values may be combined with cerebral values lower than 1 24 This means that the coronary arteries may look normal or display only slight visible changes at fairly advanced, and the cerebral arteries at greatly advanced, aortic

bral atherosclerosis more slowly, than the aortic atherosclerosis Later the coronary atherosclerosis increases at a slightly lower rate than, and the cerebral atherosclerosis almost parallel with, the aortic atherosclerosis Thus the process does not progress at a proportionally equal rate in the three arteries

It seems possible that the results of an examination of various factors' influence on the pathogenesis of atherosclerosis to some extent may depend on which artery is examined

It is emphasized that the above considerations are based on an autopsy series which is not representative of the general population, and that the results, therefore, may be valid for this series only

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wall, either directly, or through a modifying effect upon the systemic factors

Our findings clearly show that the progress of atherosclerosis is not parallel in the three arteries For instance, when aortic atherosclerosis increases linearly (Table 5, Fig 1), initially, the coronary atherosclerosis rises more rapidly, and the cerebral atherosclerosis more slowly, than the aortic atherosclerosis From an aortic value of about 1 20 the coronary atherosclerosis increases more slowly than, and the cerebral atherosclerosis almost parallel with, the aortic atherosclerosis This confirms *Baker, Iannone & Kinnard*'s (1) conclusion that atherosclerosis "does not necessarily proceed at a uniform rate in all vessels".

In conclusion it should be stressed that the above findings and considerations are based on an autopsy series, which is not representative of the general population On the contrary, the series is composed of individuals who have died from different diseases, and who may have suffered from various conditions, both of which may have influenced the progress of the atherosclerotic process If these factors influence the process at a proportionally equal rate in all arteries, the composition of the series is of minor importance with regard to the inter-relationship If not, however, the results will depend completely on the composition of the series In that case, the above considerations are valid for this series only

SUMMARY

The inter-relationship between atherosclerosis in the aorto, the coronary and the cerebral arteries has been examined in 408 individuals The total cholesterol/phospholipoid ratio in the arterial wall—the ChPh-value—has been used as an index for the degree of atherosclerosis present in the arteries

The total amount of atherosclerosis in this series was highest in the coronary arteries, slightly lower in the aorta, and considerably lower in the cerebral arteries

A close correlation was found between the amount of atherosclerosis in the three arteries, the correlation coefficient was + 0 65 for the aortic coronary relationship, + 0 58 for the aortic-cerebral relationship, and + 0 50 for the coronary-cerebral relationship Thus, a change in the degree of atherosclerosis in one artery is usually combined with a similar change in the other arteries There are, however, such great individual variations in the inter-relationship that one cannot predict the degree of atherosclerosis in one artery from the findings in another artery in a single individual

On an average, atherosclerosis appears earliest, that is at the lowest ChPh-value, in the aorta thereafter in the coronary arteries, and latest in the cerebral arteries

Compared with a linear increase of the aortic atherosclerosis, initially, the coronary atherosclerosis increases more rapidly, and the cere-

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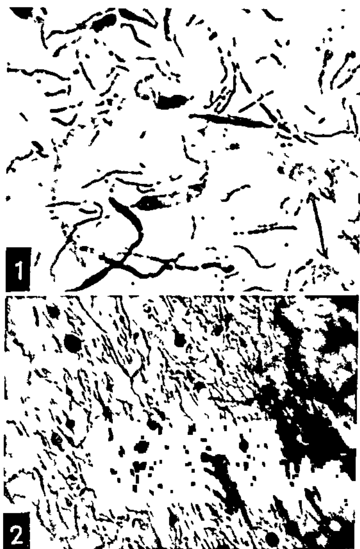
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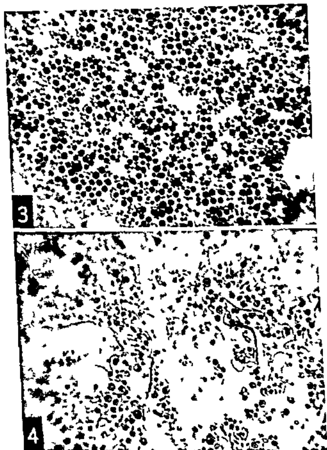
Figs 1–2

Fig 1 Strain P upon prolonged incubation (72 hours) at 37° C Note the ghost like cells Gram stain × 1350

Fig 2 Formation of the globoidal bodies in strain H Gram stain × 1000

The spherical bodies of *strain H* ranged from 4 to 6 μ They were completely rounded and could be called globoidal They maintained their round form, with minor variations in size, also after frequent parallel subcultivations at 37° C and at room temperature, for about one month Later, they began to become gradually smaller, and, in approximately three months, they reached the diameter of about 3 μ At the same time, short bacilli appeared in the cultures grown at room temperature (Fig 3), and some short filaments in the cultures grown at 37° C (Fig 4)

The macroscopic picture of the colonies of the new growth was also changed The colonies were yellowish grey in colour high convex and slower growing, in contrast to the grey, low convex and faster growing



Figs 3 4

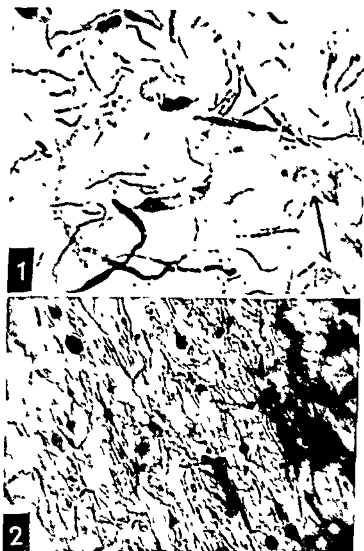
g 3 Globoidal bodies and small bacilli in room temperature culture of strain H Gram stain × 1000
g 4 Globoidal bodies and some filaments in the cultures of strain H growing at 37 C Gram stain × 1000

olonies of the primary cultures However, the organisms still spread rom the border of the colonies when grown at room temperature for a onger time (about two weeks) This spreading was observed also in the primary cultures upon prolonged growth at room temperature

Compared to the strain H another type of the changed forms was

... Fig 6) Growing at 37 C these cells occurred mostly in short chains of 2 to 7 elements (Fig 7)

A change was observed also in the macroscopical morphology of the new growth The colonies became irregular in outline high convex and



Figs 1 2

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The macroscopic picture of the colonies of the new growth was also changed. The colonies were yellowish grey in colour, high convex and slower growing, in contrast to the grey, low convex and faster growing



Fig 7

Chains of diplococcoid formations in strain P growing at 37° C Gram stain × 1000

slightly shorter than the other, so that these cells could be called geoidal (Fig 9) Besides these geoidal cells, another type of cells was found in the changed cultures of strain P growing at room temperature These cells were diplococcoid and pointed at both outer poles (Fig 10) They were more numerous in the faster growing colonies, which were found between the other, slower growing In fact, most colonies of these cultures grew very slowly After the first 48 hours of incubation, they were nearly invisible to the unaided eye They grew slightly larger within the next 24 hours of incubation but did even after prolonged incubation rarely attain the diameter of 0 5 mm The above mentioned faster growing colonies atained the diameter of 0 5 cm at the same time If subcultured small and big colonies grew from both types of the colonies

Since their appearance two years ago, the changed forms have behaved as described above Upon frequent subcultivation at 37° C and at room temperature they never reverted to the original form

In other test strains of *Bacterium anitratum*, similar bodies were induced under prolonged incubation at 37° C In some of these strains this succeeded in a much shorter time (about 3 weeks) In such cultures the spherical bodies were smaller from the beginning (Fig 11), and also the macroscopical picture of the colonies of the new growth did not differ much from that of the primary cultures of the same strains

Growth of the globoidal and of the geoidal forms in fluid media The globoidal cells grew in nutrient broth after every transfer, even of small inocula The broth was cloudy in 48 hours of growth The geoidal cells grew slower and with more difficulty in fluid media even if enriched with ascites serum or glucose When the growth of these cells was induced sometimes by repeated massive inoculations it seemed completely different from the growth of the primary cultures A soft slimy ball

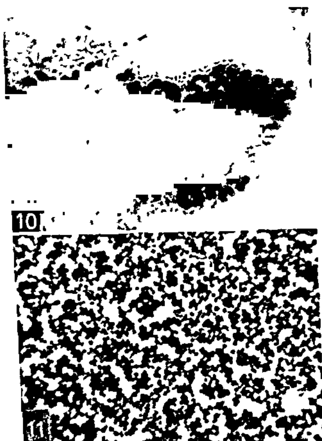


Figs 5–6

Fig 5 Spherical bodies of strain P after dividing Gram stain × 1350
Fig 6 Diplococcoid formations of strain P with short tails at the outer ends of the cells Gram stain × 1000

rubber-like in consistency, so that it was very difficult to cut them with the inoculating needle

Subcultured at room temperature, the changed cells of strain P grew in a different way They appeared no longer in chains and, presumably in accordance with this phenomenon, also their colonies were no longer so hard as when grown at 37° C However, the organisms still adhered together very tenaciously, so that it was still difficult to separate the clumps (Fig 8) into individual cells by mere grinding between two glass slides In contrast to the polymorphism in the size and shape when grown at 37° C, the changed cells of strain P were surprisingly uniform when grown at room temperature The poles of the cells were no more pointed, but rounded One of the diameters of these cells was only



Figs 10–11

Fig 10 Beside the clumps of the geoidal cells some diplococcoid cells are seen in the faster growing colonies of the changed culture of strain P growing at room temperature Gram stain × 1000

Fig 11 Small globoidal bodies of the test strain of Bacterium anitratum Gram stain × 1000

to subculture them successfully from the suspension made in sterile redistilled water Also their microscopic morphology was not changed in such a way as to indicate high osmotic sensitivity

The biochemical reactions of the globoidal and the geoidal cells, re-tested at the end of the experiments, corresponded to those of the original cultures, that is to the characteristics of *Bacterium anitratum*

DISCUSSION

In botanical vocabulary, a protoplast means the living content of a plant cell, in bacteriology it means a bacterial cell devoid of the cell wall (*Weibull* 1953) Spheroplast, however, is a bacterial cell in which the cell wall is only weakened Unfortunately, the total absence of a wall



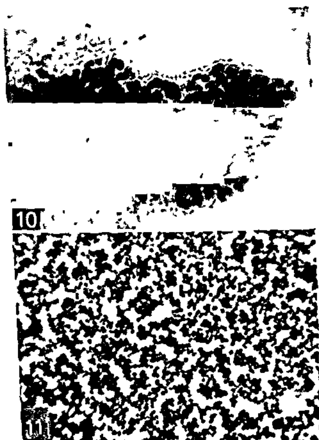
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F g 8 Clumps of the goi lal cells in strain P growing at room temperature Gram stain × 1350

Fig 9 Electron m crograph of geoidal cells Shadowed with Palladium and Platinum Magnificat on × 7 500

enlarged slowly at the bottom of the test tube the upper fluid being clear *After* prolonged incubation (from 5 to 8 days) the latter began o be opalescent and later cloudy The microscopic examination of this cloudy upper fluid showed thicl diplococcoids similar to those found in the *faster* growing colonies of the changed growth of strain P These diplococcoid cells had probably segmented themselves from the packets of the globoidal cells at the bottom of the test tube

Osmotic *stability* of the globoid il and of the geoidal cells in sterile redistilled water was found to be high The organisms preserved their colony forming capacity for 15 days During this period it was possible



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Fig 8–9

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Fig 9 Electron micrograph of geoidal cells Shadowed with Palladium and Platinum Magnification × 7 500

enlarged slowly at the bottom of the test tube the upper fluid being clear After prolonged incubation (from 5 to 8 days) the latter began o be opalescent and later, cloudy The microscopic examination of this cloudy upper fluid showed thick diplococcoids, similar to those found in the faster growing colonies of the changed growth of strain P These diplococcoid cells had probably segmented themselves from the packets of the globoidal cells at the bottom of the test tube

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might be better adapted than the parent type cells, enabled them to outgrow and replace the latter This type of changes is heritable and, as a rule, stable In the case of reverse mutation, they can revert to the original forms Under appropriate cultural conditions, these might be reselected again

SUMMARY

Upon prolonged incubation of the cultures of *Bacterium anitratum* at 37° C *i e* under conditions which induce gross polymorphism in the same and other strains of *Bacterium anitratum*, spheroplast like cells resulted which, being capable or further multiplication upon repeated subcultivation were isolated and grown in a "pure culture"

These temperature conditioned spheroplast-like cells differ from the normal bacillary forms of the same organisms in the microscopic morphology, in the picture of their colonies on solid media, in the mode of growth in fluid media and in the rate of multiplication especially in fluid media while preserving unchanged many other properties characteristic of their bacillary stage They display a remarkable stability to osmotic shock

The similarity of these cells to the spheroplasts and to the protoplasts is considered

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and its functional impairment fails to be distinguished by the criteria used Also positive functional tests of plasticity and osmotic fragility speak only for the functional impairment of the bacterial cell wall (*Lederberg & St Clair* 1958)

Impaired rigidity of their cell walls is very probably the cause of the rounding of the cells of both strains into spherical bodies

Osmotic stability of the spherical bodies differs from osmotic stability of the true protoplasts, which are extremely fragile and disintegrate immediately if not prepared in an osmotically protective solution (*Weibull* 1953), but conforms to the stability of bacterial spheroplasts The true protoplasts are defined as to lack the rigid cell wall completely and to be surrounded only by a cytoplasmatic membrane (*Pitzurra & Szybalski* 1959) In bacterial spheroplasts, residual cell wall can still be demonstrated (*Weibull* 1958) The same is the case in spheroplasts produced by the action of penicillin (*Lederberg & St Clair* 1958) *Pitzurra & Szybalski* (1959 compared the survival of spheroplasts during prolonged storage in distilled water, 0 7 per cent saline, and 20 per cent sucrose, which is said to be good for the survival of protoplasts, with the survival of normal bacillary cells under identical conditions The results indicate that the stability in these suspending media is not significantly different for the two cell forms

Also the mode of division of the spherical cells described here resembles the division of spheroplasts The geoidal bodies divide by multiple division so that morula or packet-like forms (when the segments produced are not so numerous) are seen Also the spheroplasts divide into several segments (*Pitzurra & Szybalski* 1959) Some of the segments of the geoidal cells and of the spheroplasts have a roughly bacillary shape, and, by subdivision, produce the normal forms again

Also the mode of growth of the spherical bodies in fluid media corresponds to the growth of bacterial spheroplasts in fluid media Bacterial spheroplasts are difficult to grow in fluid media If they are induced to grow, they tend to revert to the bacillary form The growth of the spheroplasts in fluid media is seen in the form of slimy streaks (*Pitzurra & Szybalski* 1959)

The mechanism of the here described spheroplasting effect of the incubation temperature is not obvious The complexity of wall components in Gram negative bacteria allows many adverse factors to pro duce wall defects (*Lederberg & St Clair* (1958) The results, however, are similar to those obtained by protoplasting factors All these phenomena appear to involve a common mechanism, *i e* the impairment of the bacterial cell wall, and also the intermediate stages, the megalomorphous phase of large swollen filaments corresponds with those obtained by the action of protoplasting factors, for example, with penicillin

Environmentally conditioned selection was the probable mechanism which isolated the cells with altered morphology into a "pure culture" of rounded bodies Serial subcultivation under conditions to which they

might be better adapted than the parent type cells, enabled them to outgrow and replace the latter This type of changes is heritable and, as a rule stable In the case of reverse mutation, they can revert to the original forms Under appropriate cultural conditions, these might be reselected again

SUMMARY

Upon prolonged incubation of the cultures of *Bacterium anitratum* at 37° C i e under conditions which induce gross polymorphism in the same and other strains of *Bacterium anitratum*, spheroplast like cells resulted which, being capable or further multiplication upon repeated subcultivation were isolated and grown in a "pure culture"

These temperature conditioned spheroplast like cells differ from the normal bacillary forms of the same organisms in the microscopic morphology in the picture of their colonies on solid media in the mode of growth in fluid media and in the rate of multiplication especially in fluid media while preserving unchanged many other properties characteristic of their bacillary stage They display a remarkable stability to osmotic shock

The similarity of these cells to the spheroplasts and to the protoplasts is considered

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and its functional impairment fails to be distinguished by the criteria used Also positive functional tests of plasticity and osmotic fragility speak only for the functional impairment of the bacterial cell wall (*Lederberg & St Clair* 1958)

Impaired rigidity of their cell walls is very probably the cause of the rounding of the cells of both strains into spherical bodies

Osmotic stability of the spherical bodies differs from osmotic stability of the true protoplasts, which are extremely fragile and disintegrate immediately if not prepared in an osmotically protective solution (*Weibull* 1953), but conforms to the stability of bacterial spheroplasts The true protoplasts are defined as to lack the rigid cell wall completely and to be surrounded only by a cytoplasmatic membrane (*Pitzurra & Szybalski* 1959) In bacterial spheroplasts, residual cell wall can still be demonstrated (*Weibull* 1958) The same is the case in spheroplasts produced by the action of penicillin (*Lederberg & St Clair* 1958) *Pitzurra & Szybalski* (1959 compared the survival of spheroplasts during prolonged storage in distilled water, 0 7 per cent saline, and 20 per cent sucrose, which is said to be good for the survival of protoplasts, with the survival of normal bacillary cells under identical conditions The results indicate that the stability in these suspending media is not significantly different for the two cell forms

Also the mode of division of the spherical cells described here resembles the division of spheroplasts The geoidal bodies divide by multiple division so that morula or packet-like forms (when the segments produced are not so numerous) are seen Also the spheroplasts divide into several segments (*Pitzurra & Szybalski* 1959) Some of the segments of the geoidal cells and of the spheroplasts have a roughly bacillary shape, and, by subdivision, produce the normal forms again

Also the mode of growth of the spherical bodies in fluid media corresponds to the growth of bacterial spheroplasts in fluid media Bacterial spheroplasts are difficult to grow in fluid media If they are induced to grow, they tend to revert to the bacillary form The growth of the spheroplasts in fluid media is seen in the form of slimy streaks (*Pitzurra & Szybalski* 1959)

The mechanism of the here described spheroplasting effect of the incubation temperature is not obvious The complexity of wall components in Gram negative bacteria allows many adverse factors to produce wall defects (*Lederberg & St Clair* (1958) The results, however, are similar to those obtained by protoplasting factors All these phenomena appear to involve a common mechanism, *i e* the impairment of the bacterial cell wall, and also the intermediate stages, the megalomorphous phase of large swollen filaments corresponds with those obtained by the action of protoplasting factors, for example with penicillin

Environmentally conditioned selection was the probable mechanism which isolated the cells with altered morphology into a 'pure culture" of rounded bodies Serial subcultivation under conditions to which they

tion against the homologous immune serum No cross-reactivity could be demonstrated between this extract and polysaccharide 263 when examined against each other's immune sera Immune serum 263 absorbed with streptococcus group A extract gave the same agar precipitation titre as non absorbed 263 serum

The teichoic acid samples were then examined against Wood 46 serum The polysaccharide A line could not be obtained with the teichoic acid preparation from strain 3528, as opposed to the preparations from strains H and A1, which both produced a strong polysaccharide A line The preparations from cell walls of *Staph albus* NCTC 7944 and *B subtilis* produced no polysaccharide A line

The agar precipitation lines obtained with the different polysaccharide preparations and the teichoic acid samples have been compiled in Table 1

TABLE 1

Agar Precipitation of Polysaccharides and Wall Teichoic Acids in 263 and Wood 46 Immune Sera

Preparation	Chemical composition			Specific polysaccharide line	
	Polyol	Sugar	Linkage	263	A
Polysaccharide 263				+	—
Polysaccharide A				—	+
Poly *Staph epidermidis* 3519				(+)	+
Poly *Staph epidermidis* 1254				—	—
Teichoic acid from					
Staph aureus H	R	Glucosamine	α and β	+	+
Staph aureus A1	R	Glucosamine	β	—	+
Staph aureus 3528	R	Glucosamine	α	+	—
Staph albus NCTC 7944	G	Galactosamine	α	—	—
B subtilis	R	Glucose	β	—	—

R = Ribitol G = Glycerol
+ (+) and — indicate strong weak or no precipitation line

Immune serum 263 was ultimately absorbed with polysaccharide A and the teichoic acid samples from strains H, A1 and 3528 Fig 2 shows clearly that wall teichoic acid from strains H and 3528 completely exhausted 263 serum for precipitating antibodies to polysaccharide 263 Polysaccharide A and the teichoic acid preparation from strain A1 did not show any absorbing capacity

Immune sera 263 and Wood 46 were also absorbed with N acetyl D glucosamine (L Light & Co) using equal volumes of undiluted serum and N acetyl D glucosamine at a concentration of 2 mg per ml in saline After incubation at 37° C for 2 hrs the titre values were examined on agar gel against polysaccharides 263 and A Exactly the same titres were obtained with absorbed and non absorbed sera Analogous results were obtained when the same sera were absorbed with glucosamine and ribitol (Adonitol Sigma)

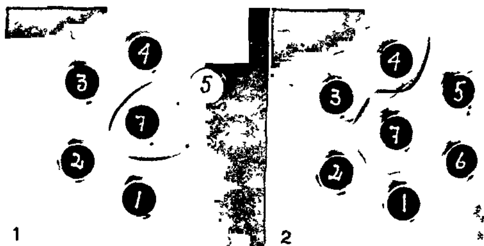


Fig 1 Cross reactivity between polysaccharide 263, 0 01 mg/ml (well 1) and ribitol teichoic acids from the *Staph aureus* strains 3528, 0 05 mg/ml (well 2) and H, 0 01 mg/ml (well 3) Polysaccharide A, 0 01 mg/ml, and ribitol teichoic acid from strain A1, 0 1 mg/ml, in wells 4 and 5 resepectively Immune serum 263 in well 7 The picture was taken after 48 hrs at 4° C. A polysaccharide A line was just visible close to well 7, between this and wells 3 4 and 5, after 24 hrs of incubation

Fig 2 Immune serum 263 absorbed with ribitol teichoic acids from the *Staph aureus* strains A1 (well 3), H (well 5) and 3528 (well 6), and with polysaccharide A (well 2) Unabsorbed 263 serum in wells 1 and 4 polysaccharide 263 in well 7 Excess antigen was used for absorption which was performed directly in the basins The picture was taken after 48 hrs at 4° C

EXPERIMENTAL PROCEDURES AND RESULTS

Precipitation

Purified polysaccharide 263 was precipitated in 263 immune serum by the ring test and on agar diffusion in dilutions up to 1 10^6 The precipitation was unaffected following treatment of the serum with mercaptoethanol

Varying concentrations of the ribitol teichoic acid samples from the cell walls of the *Staph aureus* strains H, A1 and 3528 were compared by agar precipitation with the polysaccharides 263 and A, all against a potent 263 serum The teichoic acid preparations from strains H and 3528 both produced a distinct polysaccharide 263 line (Fig 1) No such line was obtained with various dilutions of polysaccharide A and the teichoic acid preparation from strain A1

No polysaccharide 263 line was produced by the ribitol teichoic acid preparation from *B subtilis*, by glycerol teichoic acid from cell walls of *Staph albus* NCTC 7944, or by purified polysaccharide from the *Staph epidermidis* strain 1254 A very weak polysaccharide 263 line was sometimes produced by high concentrations of the polysaccharide preparation from strain 3519 No polysaccharide 263 line was produced by staphylococcal α toxin or by preparations of protein A and SPA Streptococcus group A extract produced a distinct band of precipita-

tion against the homologous immune serum No cross reactivity could be demonstrated between this extract and polysaccharide 263 when examined against each other's immune sera Immune serum 263 absorbed with streptococcus group A extract gave the same agar precipitation titre as non absorbed 263 serum

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In another experiment, 25 μg wall teichoic acid from strain H was absorbed with 0 25 ml of undiluted Wood 46 serum After incubation, the absorbed preparation was examined on agar gel against Wood 46 and 263 sera Whilst no polysaccharide A line could be demonstrated, the specific polysaccharide 263 line was obtained to the same titre from the absorbed preparation as with non-absorbed teichoic acid Inverse results were found when serum 263 was used for absorption of the same teichoic acid, *i e* the absorbed preparation produced no polysaccharide 263 line, while the polysaccharide A line was obtained in a titre similar to that of the non-absorbed teichoic acid Analogous findings were made using a crude polysaccharide preparation from strain 263 producing both polysaccharide lines

Complement Fixation

Serial dilutions of immune serum 263 were examined for complement-binding antibodies against serial dilutions of polysaccharide 263, starting with a concentration of 0 1 mg per ml of polysaccharide 263 Positive reactions were obtained with dilutions of the antigen up to 1 20480 A marked postzone phenomenon was observed with the highest concentrations of the antigen

TABLE 2
Complement Binding Antibodies in Immune Sera 263 and Wood 46

Antigen		Reciprocal titre values in sera	
		263	Wood 46
Polysaccharide 263	0 5 μg/ml	160	—
Polysaccharide A	0 1 μg/ml	80	160
Teichoic acid *Staph aureus* 3528	1 6 μg/ml	320	—
Teichoic acid *Staph aureus* A1	1 6 μg/ml	40	320

In another experiment, the complement-binding property of polysaccharide 263 was compared with that of polysaccharide A and wall [illegible] 3528 and A1 Table 2 shows clearly that the [illegible] lysaccharide 263 and wall teichoic acid from strain [illegible] that of polysaccharide A and wall teichoic acid from strain A1

When immune sera to our type strains were examined for complement-binding antibodies against polysaccharide 263 positive reactions were only obtained with sera giving the polysaccharide 263 line

Agglutination Inhibition

Two immune sera 263, diluted 1 5, were absorbed with equal volumes of a saline solution of polysaccharide 263 at a concentration of 0 1 mg per ml The absorbed sera were then examined for agglutinating anti-

bodies by tube agglutination, using suspensions of strain 263 and the type strains as antigen. For all strains, the titres obtained were the same as with non-absorbed sera.

In a similar experiment, the factor 263-1, 263-2, *m* and *as* sera were absorbed with polysaccharide 263. No absorption was obtained.

The absorbed sera produced the polysaccharide 263 line against non-absorbed 263 serum, showing that excess antigen had been used in the above experiments.

Indirect Haemagglutination

Polysaccharide 263 did not sensitize normal sheep cells. Sensitization however, was obtained using tanned cells. The sensitized erythrocytes were agglutinated by dilutions of 263 sera up to 1 : 20480.

Haemagglutinating antibodies against the sensitizing substance of the polysaccharide 263 preparation were found in most immune sera studied. The titres obtained with sera to some of our type strains are listed in Table 3. For comparison the titres obtained with tanned cells sensitized with polysaccharide A are included.

TABLE 3

Haemagglutination of Sensitized Tanned Sheep Erythrocytes in some Staph. aureus Immune Sera

Sensitizing antigen	Reciprocal titre values in sera										
	1503	364	F21	17A	3189	2095	Wood 46	Cowan I	Cowan II	Cowan III	263
Polysaccharide 263	10	5120	—	640	40	10240	80	80	40	20480	20480
Polysaccharide A	2560	80	2560	5120	20480	320	20480	5120	10240	640	1280

TABLE 4

Haemagglutination of Sensitized Tanned Sheep Erythrocytes in Absorbed Immune Sera.

Sensitizing antigen	Serum 263 absorbed with		Serum Wood 46 absorbed with	
	Poly 263 STSC	Poly A STSC	Poly 263 STSC	Poly A STSC
Polysaccharide 263	—	5120*	—	(10)
Polysaccharide A	640	—	20480	—

Serum titres before absorption are given in Table 3.
* = Reciprocal titre values STSC = Sensitized tanned sheep cells

The results obtained indicated that the sensitizing substance of polysaccharide 263 was different from that of polysaccharide A. This suggestion was confirmed by cross-absorption experiments (Table 4). Each absorption was performed using 0.25 ml of packed, sensitized cells for absorption of 1 ml of immune serum diluted 1 : 2. The heterologous

sera were completely absorbed after 3 successive absorptions, whilst the homologous sera had to be absorbed 13 times for the complete removal of haemagglutinating antibodies

In another experiment, the polysaccharide 263 preparation was absorbed with tanned cells While the sensitizing substance was completely removed, the absorbed preparation reacted with 263 serum on agar precipitation and in the complement fixation test to the same titre as non-absorbed preparations

Tanned sheep erythrocytes were also treated with ribitol teichoic acid from strain 3528, using 0 2 mg of the teichoic acid preparation for sensitization of 0 1 ml of packed cells Sensitization was not obtained

In another experiment the polysaccharide 263 preparation was subjected to paper electrophoresis for 3 hrs in N acetic acid, using a potential of 17 V/cm The precipitinogen, which had migrated towards the anode (19), was eluted with water and used for sensitization of tanned sheep cells Sensitization was readily obtained, showing that a separation of the erythrocyte-sensitizing and the precipitating antigen had not been achieved

The antigenicity of the sensitizing principle of the polysaccharide 263 preparation was examined by immunizing rabbits with sensitized sheep erythrocytes The immunization was carried out as described by *Oeding et al* (26) Before use, the serum was absorbed with normal sheep cells The absorbed immune serum (polysaccharide 263 STSC serum) agglutinated tanned sheep cells sensitized with polysaccharide 263 to a titre of 1 1280 Precipitating or complement-binding antibodies could not be demonstrated No haemagglutination was obtained with a mercapto-ethanol-treated sample of the immune serum

The sensitivity to trypsin of the antigen sensitizing tanned sheep cells was examined in several experiments 0 1 mg of the polysaccharide 263 preparation was incubated with 0 01 to 0 1 mg of crystalline trypsin at various pH The digested samples, together with samples of polysaccharide 263 treated in the same way with saline or buffers, were then used for sensitization of tanned sheep erythrocytes While the cells sensitized with buffer or saline treated polysaccharide agglutinated with strong reactions to a titre of 1 640 or 1 1280 in polysaccharide 263 STSC serum, the titres obtained in the same serum with tanned cells sensitized with trypsin-treated polysaccharide 263 varied from zero to 1 640, and the reactions were generally weaker The possibility that these variable and rather inconclusive results were due to an unspecific or blocking effect of trypsin on the tanned cells, was examined by treating the tanned sheep cells with trypsin before sensitization with undigested polysaccharide 263 This treatment was followed by a slight reduction both in titre value and in strength of the reactions

In one experiment, a trypsin treated sample of polysaccharide 263 was subjected to paper electrophoresis for 3 hrs in N acetic acid, pH 2 35, applying a voltage of 17 V/cm The distance of migration was

exactly the same as that of an untreated control Hydrolysed samples of the eluted polysaccharide were examined by paper chromatography in n butanol acetic acid water (4 1 1) Glycine, alanine, lysine, glutamic acid and glucosamine were revealed by ninhydrin The contents of the amino acids and glucosamine in the eluates of the trypsin treated and the untreated samples of polysaccharide 263 appeared to be similar

A trypsin treated sample of the polysaccharide 263 preparation was examined for the presence of free amino acids A mixture of trypsin-treated polysaccharide and Amberlite IR-120 was allowed to stand for 3 hrs, whereafter the washed resin was eluted with 5 N ammonia The evaporated and redissolved eluate was examined chromatographically in n butanol acetic acid-water (4 1 1) With the exception of a trace of lysine, no amino acids had been released during the treatment with trypsin

Following autoclaving of a saline solution of the polysaccharide 263 preparation at 120° C for 1 hr, the sensitizing ability was completely lost The autoclaved preparation reacted, however, on agar precipitation and in the complement fixation test to the same titres as the untreated polysaccharide

Free amino acids were not found when the autoclaved sample was examined chromatographically The autoclaved sample was also examined by paper electrophoresis in N acetic acid, applying a voltage of 17 V cm The migration after 3 hrs was the same as that of an untreated control The eluted polysaccharide was hydrolysed in 6 N hydrochloric acid for 16 hrs and examined by paper chromatography in n butanol acetic acid water (4 1 1) Spots corresponding to glycine, alanine, lysine, glutamic acid and glucosamine were demonstrated on the ninhydrin treated chromatogram The colour intensity of the spots indicated that similar amounts of the amino acids and of glucosamine were present in the eluates of the autoclaved and the untreated samples of polysaccharide 263

The Distribution of the Polysaccharide 263 Precipitinogen among Staphylococcal strains

A strong polysaccharide 263 line was produced by suspensions of the variant strains obtained by *in vitro* lysogenization of strain 263 (18) The suspensions of two of the phage variants, strain 263–KS6–3 and 263 KS6–6, failed to produce the polysaccharide A line against 263 and Wood 46 serum However, a very weak polysaccharide A line was demonstrated when concentrated extracts of live 263–KS6–3 and 263–KS6-6 microbes were used for agar diffusion

A polysaccharide 263 band was produced by extracts or suspensions [illegible]

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the exception of strains Wood 46 and 670, were able to exhaust immune serum 263 for precipitating antibodies against polysaccharide 263

The polysaccharide 263 line was found with suspensions of the *Staph aureus* strains Oxford, H, Copenhagen and *Kapral & Lie*'s 18 Z strains (21). The band produced by strains H and Copenhagen was very weak as opposed to a strong polysaccharide A band Our stock strain 209 (Copenhagen) produced no polysaccharide 263 line Neither did any of the three Smith strains (20) Strain 3528 produced a strong polysaccharide 263 line The polysaccharide A line, on the other hand, could not be demonstrated when 3528 microbes were examined against 263 and Wood 46 immune sera

The polysaccharide 263 line was produced by 40 out of 45 staphylococcal strains within phage group I All 45 strains gave a protein A line (9) with pooled human serum, and the polysaccharide A line was produced by most of them against rabbit immune serum

Suspensions of live microbes of 40 coagulase positive staphylococcal strains, representing the phage groups II, III,type 187 and the mixed group, and non-typable strains, were examined for production of the polysaccharide 263 line The line was produced by most strains within phage group III, and by some strains within the other groups, non-typable strains included

The polysaccharide 263 line was not found when 100 coagulase negative staphylococcal strains were examined against immune serum 263 Forty of the strains, however, produced one or two (a few strains) other bands of precipitation with 263 serum These lines were not examined further

DISCUSSION

The serological examination of polysaccharide 263 revealed that the purified preparation, besides producing a specific agar precipitation line, also exhibited complement-binding and erythrocyte sensitizing properties The serological reactivity was high for all three types of reaction

The agglutination inhibition experiments suggest that polysaccharide 263 is no agglutinogen, or at least, not exposed to the surface as an agglutinogen It is clear, therefore, that this polysaccharide antigen cannot be responsible for the strong cross-agglutination of *Staph aureus* strains in rabbit immune sera The same conclusion was reached by *Haukenes* for polysaccharide A (14) Nor is polysaccharide 263 identical to the a_3, *m*, 263-1 and 263 2 antigens, *i e* the known type agglutinogens of strain 263 (17)

The production of the specific polysaccharide 263 line by wall teichoic acids from the *Staph aureus* strains H and 3528 (cf Fig 1) shows that the common N-acetylglucosaminylribitol grouping (the amino sugars in bacterial cell walls occur as acetyl derivatives) or part of it, is responsible for the serological reactivity on agar precipitation Glucosyl-

ribitol teichoic acid from *B subtilis* and streptococcal group A carbohydrate, the serological specificity of which is determined by side chains of N acetylglucosamine (23), did not cross-react with polysaccharide 263 This shows that neither ribitol nor N-acetylglucosamine alone are responsible for the precipitating activity of polysaccharide 263 but points to the importance of the whole N acetylglucosaminylribitol unit The importance of this unit is evident from the results obtained when the teichoic acid preparations from strains H, 3528 and A1 were also examined against Wood 46 serum, and from the results of the absorption experiments shown in Fig 2 While the teichoic acid preparation from strain A1, containing β-linked N-acetylglucosaminyl residues only, produced a strong polysaccharide A line, no such line was produced by the teichoic acid preparation from strain 3528, which contained exclusively (or almost exclusively) α linked N-acetylglucosaminyl residues Furthermore, the precipitating antibodies against polysaccharide 263 were completely absorbed by the teichoic acid preparation from strain 3528, whilst no absorption was obtained with the preparation from strain A1 The conclusion to be drawn from the experiments concerning the chemical basis of the precipitating activity of polysaccharide 263 is therefore that the N-acetylglucosaminylribitol unit is the immunological determinant of this polysaccharide and of polysaccharide A, and that the specificity of the antibodies formed dep...

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The importance of the configuration of the N acetylglucosaminyl ribitol unit for the serological specificity of wall ribitol teichoic acids from *Staph aureus* has earlier been pointed out by *Sanderson et al* (27) and *Nathenson & Strominger* (25) These authors, however, reached this conclusion from studies of the inhibitory action of wall teichoic acids and their constituents on the agglutination of staphylococcal cell walls ...

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The results obtained by absorption of the teichoic acid preparation from strain H with Wood 46 and 263 sera suggest that teichoic acid preparations producing both polysaccharide lines are mixtures of serologically different polymers Some polymers are built up of α N acetylglucosaminylribitol units, and others of β N-actylglucosaminylribitol units A mixture of both configurations within the single polymer (27) does not seem to exist The wide distribution of polysaccharide 263 and polysaccharide A (14) indicate that both ribitol teichoic acid polymers are found in most strains of *Staph aureus*

The positive complement fixation obtained with polysaccharide 263 and ribitol teichoic acid from strain 3528 in 263 serum is, most likely,

down in the centrifuge at room temperature, washed twice with basal medium and resuspended in the basal medium From this suspension 0 5 ml was inoculated into 5 ml of basal medium When this culture had entered the logarithmic growth phase, usually after 4 to 6 hours, it was supplemented with 0 5 ml DNase and penicillin to a final concentration of 2000 units per ml Incubation continued for another 1½ h Next the culture was spun down in the centrifuge and the pellet was resuspended in 5 ml of basal medium plus 0 2 ml of penicillinase (Difco) The culture was left for 15 minutes at 37° Dilutions were next prepared and plated on complete media This was called "Penicillin selection 1" The rest of the culture was again centrifuged, resuspended in basal medium plus growth factor This culture was incubated for another 48 h and went through a procedure similar to that described above This was called "Penicillin selection 2" Each experiment performed in order to recover nutritionally deficient mutants after UV treatment usually consisted of 2 to 3 successive penicillin selections according to this procedure

The colonies which appeared on the blood agar plates after the penicillin selections were replicated on basal agar plates Colonies which did not grow on the basal plates were picked and checked with regard to growth requirements by streaking on basal media and on media supplemented with various growth factors

This technique has been successfully used to obtain the following amino acid auxotrophs

Single markers

met, orn (alternative growth with cit and arg), *arg, phe, ilva, his, thr, pro*

Double markers

arg ilva, his pro, his gly

The strains carrying double markers were obtained from singly marked strains in separate experiments

When the present technique was evolved parallel experiments were performed with *E coli* K12 The number of mutants obtained from the Enterobacterium is in striking contrast to the very few ones recovered from *Neisseria meningitidis* This difference between the two bacterial systems will be commented upon in following communications from this laboratory (13, 14)

Behaviour of Auxotrophs in Mixed Cultures

One requirement which should be fulfilled by auxotrophs to be used in genetic experiments is that any two strains carrying different nutritional markers must not be able to imitate the behaviour of genetic recombinants when occurring in mixed cultures

TABLE 2

Satellite Growth of some Auxotrophs from Neisseria meningitidis

Exper no	Auxotroph used as donor of amino acid	Composition of test plate	Auxotrophs used as acceptor of amino acid					
			his	thr	pro	arg ilva	his pro	his gly
1	his	A + his	++	—	—	—	—	—
2	thr	A + thr	—	++	—	—	—	—
3	pro	A + pro	+	—	++	—	—	—
4	arg ilva	A + arg + ile + val	+	—	—	++	—	—
5	his pro	A + his + pro	++	trace	++	—	++	—
6	his gly	A + his + gly	++	trace	—	—	—	++

The acceptor strain was spread on the test plate The donor strain was cross streaked
The cultures were observed for satellite growth after 1 2 and 3 days
Growth response indicated by + and ++

TABLE 3

Mutual Cross Feeding in Mixed Cultures of Auxotrophs from Neisseria meningitidis

Exper no	Auxotroph tested	Test medium	Auxotroph tested in mixed culture Growth response					
			his	thr	pro	arg ilva	his pro	his gly
1	his	A	—	+	++	—	—	—
2	thr	A	++	—	+	++	+	++
3	pro	A	++	++	—	—	—	++
4	arg ilva	A	—	+	—	—	—	++
5	his pro	A	—	++	—	—	—	—
6	his gly	A	—	++	++	++	—	—

All strains used were incompetent f...
ly grown in basal media
washed for removal of g
tely 10⁹ mill per ml w
days was restreaked or
symbiotic system was cl
complete ... and examination of single colonies
No recombination could be demonstrated

Several nutritionally deficient mutants were studied in mixed cultures in order to elucidate this problem For these experiments mutants were selected which are negative with regard to competence of transformation (11)

From the data presented in Table 2 it is seen that cross feeding may take place between certain auxotrophs of N meningitidis when tested in the "satellite technique" A cross-feeding is of course to be found in most bacteria when searched for, and the cross-feeding found among the present auxotrophs of *N meningitidis* is no more pronounced than the one found in experiments with *E coli* mutants using the same technique Among the auxotrophs tested only a his mutant was cross-fed to any significant extent using this technique

down in the centrifuge at room temperature, washed twice with basal medium and resuspended in the basal medium From this suspension 0 5 ml was inoculated into 5 ml of basal medium When this culture had entered the logarithmic growth phase, usually after 4 to 6 hours, it was supplemented with 0 5 ml DNase and penicillin to a final concentration of 2000 units per ml Incubation continued for another 1½ h Next the culture was spun down in the centrifuge and the pellet was resuspended in 5 ml of basal medium plus 0 2 ml of penicillinase (Difco) The culture was left for 15 minutes at 37° Dilutions were next prepared and plated on complete media This was called "Penicillin selection 1" The rest of the culture was again centrifuged, resuspended in basal medium plus growth factor This culture was incubated for another 48 h and went through a procedure similar to that described above This was called "Penicillin selection 2" Each experiment performed in order to recover nutritionally deficient mutants after UV treatment usually consisted of 2 to 3 successive penicillin selections according to this procedure

The colonies which appeared on the blood agar plates after the penicillin selections were replicated on basal agar plates Colonies which did not grow on the basal plates were picked and checked with regard to growth requirements by streaking on basal media and on media supplemented with various growth factors

This technique has been successfully used to obtain the following amino acid auxotrophs

Single markers
met, orn (alternative growth with cit and arg), *arg, phe, ilva, his, thr, pro*
Double markers
arg ilva, his pro, his gly

The strains carrying double markers were obtained from singly marked strains in separate experiments

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One requirement which should be fulfilled by auxotrophs to be used in genetic experiments is that any two strains carrying different nutritional markers must not be able to imitate the behaviour of genetic recombinants when occurring in mixed cultures

When a selective pressure is established in such a system by a plating on basal medium a very high number of mixed colonies may be isolated Under these conditions we find a complete imitation of a genetic recombination system as the one used for instance in *E coli* K12 (5)

DISCUSSION

Auxotrophs must fulfil certain requirements in order to be used as markers in genetic cross A number of experiments have been performed to elucidate these problems with regard to the auxotrophs described in the present communication These experiments were performed with nutritionally deficient mutants which are incompetent of transformation by deoxyribonucleates (11)

One requirement is that all colony forming units which are genetically equipped to grow in a selective system should be phenotypically expressed as a colony In the first part of this paper experiments have been presented which demonstrate that this requirement is only fulfilled under certain conditions

The population of individual units in a culture of *N meningitidis* is usually heterogeneous with regard to requirements in order to initiate growth The heterogeneity is most pronounced in cultures grown on the surface of agar media An analysis of colony forming capabilities shows that each colony which grows on the surface of the basal medium contains a high number of individual units which must be supplemented in order to initiate growth on the basal medium

The heterogeneity in question is not gentic but phenotypic since all colonies which appear those on blood agar plates as well as those on basal plates grow well when streaked on basal plates and appear genetically identical upon further examination

The degree of heterogeneity seems to be related to the growth phase of a fluid culture In the middle of the logarithmic growth phase the heterogeneity seems to be least pronounced

The nature of this heterogeneity is not at all clear but it could be taken to indicate a pronounced interdependence between the individual units within the culture Some enzyme system or systems required for growth on the basal medium may only be phenotypically expressed in part of the population The rest of the population could grow by cross feeding A derepression or induction with resynthesis of the required enzymes might not be possible in the latter part of the population when plated on the basal medium The enzymes in question might for instance include the ones which are necessary for the assimilation of essential metabolites or the production of key intermediates

One aspect of the heterogeneity is the problem of recovery of colony forming units on basal media Apparently this problem may be solved by the use of a more complex defined medium such as that described by Catlin & Schloer (3) Such medium without glucose might also

The type of experiment reported in Table 3, however, gives a picture which is not obtained with mutants from *E coli* Clearly, two different meningococcal auxotrophs may grow on the basal medium as the result of a mutual cross-feeding In order to start a cross-feeding the two strains must obviously get into intime contact Once established such symbiotic systems may be subcultured by streaking on the basal medium with full growth

The ease with which such complementation of growth requirements can be established in *N meningitidis* is indeed extraordinary It is also clear, however, that certain pairs of mutants cannot easily complement each other

A symbiotic system may be resolved at any time Upon spreading on complete media the original partners are recovered by picking single colonies However, among the single colonies picked, usually between 1 and 5 per cent still contain a mixed population which must be resolved by repeated isolation of single colonies on complete media Thus, the control of the symbiotic systems seems to indicate that a cross-feeding can occur within the colony forming unit The question was accordingly raised whether the two auxotrophs may actually combine in order to establish a colony-forming unit which develop into a mixed colony

The data compiled in Table 4 demonstrate that mixed colonies are readily formed on blood agar plates when two cultures of *N meningitidis* are mixed and incubated together for 1 to 2 hours When a mixed colony is subcultured and spread anew, 1 to 5 per cent mixed colonies may still be present True recombinants, however, have never been isolated from mixed cultures of strains which are incompetent of transformation (10, 11)

TABLE 4

Mixed Colony Forming Units in Cultures of Neisseria meningitidis

Exper no	Auxotrophs used		Medium used for isolation of single colonies	Homogeneous colonies	Mixed colonies	Recombinant colonies
	Strain 1	Strain 2				
1	his	pro	Blood Agar Plates	93	7	0
2	his	pro	Blood Agar Plates	98	2	0
3	his	pro	Blood Agar Plates	99	1	0
4	his	pro	Blood Agar Plates	96	4	0
5	his	pro	Blood Agar Plates	99	1	0
6	his	pro	Blood Agar Plates	97	3	0
7	his	pro	Basal Agar Plates	0	100	0
8	his	pro	Basal Agar Plates	0	58	0

The test strains are incompetent of transformation (11) The auxotrophs were grown separately in fluid media supplemented with their respective growth factors to logarithmic growth Equal parts of the cultures were mixed left for 2½ h at 37° diluted in saline and spread on blood agar plates or on basal agar plates Single colonies were picked and streaked on basal plates and blood agar plates Each colony was checked for the presence of mixed population homogeneous population or true recombination All mixed colonies from blood agar plates grew symbiotically on the basal medium

When a selective pressure is established in such a system by a plating on basal medium, a very high number of mixed colonies may be isolated. Under these conditions we find a complete imitation of a genetic recombination system as the one used for instance in *E. coli* K12 (5).

DISCUSSION

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appear logical always considering the glucose effect on adaptive enzyme synthesis Even with the basal medium used in the present studies, however, a number of colonies equal to that found on blood agar plates may be obtained during all growth phases provided small amounts of complete medium are added Upon the addition of 1 per cent Heart Infusion Broth, auxotrophs form microcolonies which are hardly visible while prototrophs develop into complete colonies (15)

If the heterogeneity is due to a tendency to develop interdependence in meningococcal cultures the behaviour of auxotrophs might be expected to reflect the phenomenon

Several experiments performed with mixed cultures of various auxotrophs indeed support the assumption that an interdependence is readily established between the units in an *N meningitidis* culture Two mutants with different growth requirements may in mixed culture grow symbiotically on the basal medium In order to establish such a complementation the mutants must obviously get into close contact

Such cross-feeding is evidently established even on the level of the colony-forming unit When mutants with different nutritional markers are mixed and plated for the isolation of single colonies on complete media, mixed colonies are found Whether this formation of mixed colonies is due to aggregation or to a real exchange of partners between individual diplococci is not known

The experiments with nutritionally marked mutants show no evidence of genetic recombination in mixed cultures of *N meningitidis* provided both strains are incompetent of transformation The problem of competence is treated in following communications (11)

Evidently the "auxotrophs complementation" described in the present communication must greatly affect any screening system based on the isolation of recombinants in genetic cross The nutritionally defect mutants of *N meningitidis* may therefore not lend themselves to a wide variety of genetic experiments based on the principles used in the genetics of *E coli*

Auxotrophs are not easily isolated from *N meningitidis* by a technique in accordance with the principles successfully employed in the *E coli* system (4, 5) Several aspects of the problems concerned will be treated in some detail in other communications from this laboratory (13, 14, 15)

The difference between the Neisseria system and that of *E coli* may on the one hand be due to a difference in the mutagenic effect of UV light on the two microbes On the other hand the reason might be a lack of selective penicillin effect in the case of meningococci

There is indication that UV treatment in fact is an ineffective mutagen in *N meningitidis*, and that the mutants actually isolated by the present technique in fact are spontaneous ones (13) This would explain why two or three successive penicillin selections were regularly necessary in order to obtain mutants Why UV light should lack muta-

genic effect on meningococci is not known Since the lethal effect is very high in this microbe (13) the reason may be that the lethal dose and the mutagenic dose are not sufficiently different to permit a differentiation between the two effects In this connection it may be relevant to mention that hydroxylamine has a killing effect on meningococci which is too high to permit the use of this chemical as a mutagen Hydroxylamine is an adequate mutagen in *E coli* (12)

The desired penicillin effect is a selective killing of prototrophs, or rather metabolizing cells A lack of effect might result from survival of prototrophs or from the synchronous killing of auxotrophs

There are experimental results which indicate that a difference actually exists between the effect of penicillin on *N meningitidis* and *E coli* (14) Metabolizing cells of *E coli* in basal media are rapidly killed by penicillin, while under the same conditions a high number of *N meningitidis* units survive for many hours Thus the effect of penicillin seems to be bacteriostatic on a high number of the colony-forming units in the Neisseria culture It would be tempting to draw a parallel between this effect and the tendency of meningococcal cultures to develop interdependence as has been postulated above Under the growth conditions used in the penicillin experiments the population is heterogeneous This might mean that once a certain part of the population has been killed the rest cannot grow unless supplemented This would of course result in a simulation of a bacteriostatic system That the cultures actually contain a high number of colony forming units which must be supplemented has been demonstrated in the growth experiments reported

Even if a favourable bacteriocidal system is established however, this does not necessarily mean that the penicillin killing is selective The ease with which interdependence is established between individual units in mixed cultures of meningococcal auxotrophs indicates that auxotrophs may easily be killed as the consequence of cross feeding Since the cross feeding is established also within the colony forming units even a high dilution cannot result in a selective killing

Thus the tendency towards a development of cross feeding in meningococcal cultures may be assumed to result in a survival of prototrophs as well as in a killing of auxotrophs in the presence of penicillin

SUMMARY

A defined medium has been used for the study of mutants from *Neisseria meningitidis* Auxotrophs have been isolated by a modified penicillin technique

A fraction of the colony forming units present in a genetically homogeneous culture of *Neisseria meningitidis* must be supplemented in order to initiate growth on the basal medium The size of this fraction

depends on the medium from which the culture is harvested and on the growth phase

When mutants carrying different nutritional markers come into contact colony forming units are established which develop mixed colonies Such colonies may grow on the defined medium by a mutual cross-feeding No genetic recombination takes place in mixed cultures when both of the test strains are incompetent of transformation

It has been postulated that a pronounced interdependence regularly is established between individual units in cultures of *Neisseria meningitides*

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TRANSFORMATION OF AUXOTROPHS OF *NEISSERIA MENINGITIDIS*

By

K JYSSUM and S LIE

Received 14 ix 64

It has been established that nutritionally deficient mutants can be isolated from cultures of *Neisseria meningitidis* by a modified penicil lin technique (11)

Several auxotrophs carrying different nutritional markers effectively establish mutally cross feeding systems when they get into contact Such symbiotic systems grow well on basal media However, no genetic recombination has been demonstrated in cross feeding systems when the test strains are incompetent of transformation (11 12)

In the present paper two problems have been discussed The first is concerned with transformation of nutritionally defect mutants of *Neisseria meningitidis* by deoxyribonucleates The second deals with mero mixis of meningococci in cultures

The problem of competence will be treated in following communica tions (12 13)

MATERIALS AND METHODS

The methodology and experimental manipulations used in this investigation were analogous with those previously described (11)
Some additional procedures and modifications have been described in the text

RESULTS

Transformation of Auxotrophs by Deoxyribonucleates

In the present studies the following competent receptor mutants isolated from the meningococcus strain M1 (11) were employed

Aux 12 his	Aux 21 gly
Aux 14 thr	Aux 18 his gly
Aux 17 pro	

The competence (12 13) was judged by the faculty of the strains to be transformed from str s to str r

The first question to be attacked was whether these strains could be transformed from auxotrophy to prototrophy The technique which was used in these experiments has been described in the legend to Table 1

TABLE 1

Transformation of some Auxotrophs of Neisseria meningitidis

Auxotroph tested	DNA used in transformation	Transformations per ml		Transformation frequency	
		Selective medium	Transformations	Transformations per exposed unit	Ratio str-r nutritional marker
12 his	M1 prot. str-r	A	5.3×10^{3}	3.5×10^{-5}	0.34
"	"	str	1.8×10^{3}	1.2×10^{-5}	–
14 thr	"	A	3.0×10^{2}	6.3×10^{-6}	1.75
"	"	str	5.5×10^{3}	1.1×10^{-5}	–
17 pro	"	A	4.1×10^{5}	8.5×10^{-3}	0.75
"	"	str	2.8×10^{5}	6.4×10^{-3}	–
21 gly	"	A	2.6×10^{5}	1.3×10^{-3}	0.67
"	"	str	3.6×10^{5}	1.8×10^{-3}	–
18 his gly	"	A	3.2×10^{4}	1.4×10^{-4}	10.72
"	"	his	9.4×10^{5}	3.8×10^{-3}	0.39
"	"	gly	6.2×10^{5}	2.6×10^{-3}	0.58
"	"	str	3.5×10^{5}	1.5×10^{-3}	–

Receptor strains were grown in fluid basal media until middle logarithmic growth phase. The transformation system contained 0.3 ml of heart infusion broth (HIB) with 0.01 ml 0.5 M $CaCl_2$ per ml, 0.1 ml of donor DNA with approximately 5 µg of DNA in Zamenhof buffer and 0.1 ml of the receptor culture. The mixture was incubated at 37°. DNase was added after 45 minutes, and after another 15 minutes at room temperature dilutions were prepared and plated on selective media and complete media. Controls were included in which the DNA had been treated with DNase before addition. Phenotypic expression of the str-r marker was allowed to take place for 6 hours (3). The screening dose of streptomycin was 100 µg per ml medium.

Evidently, all the receptor strains may easily be transformed when screened for the transformation of one marker at a time. The Aux 18 his gly could be transformed to prototrophy, to his gly^{+} and to his^{+} gly^{-}.

In these experiments no attempts were made to obtain maximal frequency of transformation (13). In separate experiments, however, the receptor strains were followed through the various growth phases in fluid cultures. No high or well defined wave of competence could be demonstrated. This seems to agree with previous findings by *Catlin* (4). A study of the time in which the cells had to be exposed to DNA showed a significant increase in the yield of transformants during a period of 30 to 45 minutes. An incubation time of 45 minutes was used in the following experiments. The transformation frequencies are on the whole low. From the data presented in Table 1 it is seen that the receptor strains seem to fall into two groups since the auxotrophs 12 and 14 have lower frequencies than the auxotrophs 17, 21 and 18. For each nutritional marker the transforming frequency obtained was related to that of the str-r marker. These calculations emphasize a difference between the thr marker of Aux 14 and the other nutritional markers analysed. It may be seen that the thr marker is in a position between the other single markers and the double marker his gly with respect to its relative transformation frequency.

In the mutant Aux 18 his gly, both markers may be transformed at

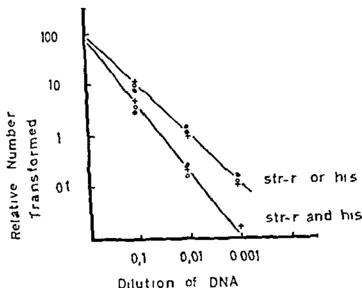


Fig 1

The effect of DNA concentration on the relative numbers of single and double transformations —Double transformations were scored on cells transformed to str r The technique has been described in the text

the same time either by an unlinked, double transformation, or by a linked transformation This observation raised the problem of linkage among the markers under study A number of experiments were performed in order to obtain some information

Previous studies have shown that incompetent variants of the present auxotrophs effectively establish mutally cross feeding systems in mixed cultures (11) Thus a selective system based on the recovery of recombinants from transformation of auxotrophs must be used with precaution A transformation in one partner of a colony-forming unit or different transformations in separate partners within the colony-forming unit might be expected to result in mixed colonies in which a part of the population grow by cross feeding Such occurrence may indeed be demonstrated When the mutant Aux 18 his gly was transformed with saturating concentrations of DNA from Prot M2 55 per cent of the colonies which appeared on his plates had also received the his allele Upon spreading of the colonies and single colony isola tion however it became apparent that only 4 per cent consisted of a homogeneous population of prototrophs The rest of the colonies contained a mixed population of his gly⁺ and his⁺ gly units This is taken to indicate that in these colonies different transformations had occurred in the partners of the colony forming unit

Less ambiguity might be expected from selective systems based on drug resistance markers The addition of the drug should result in a

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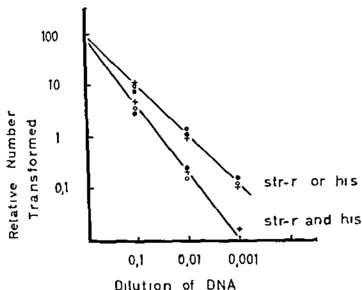


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during an observation time of 8 hours. The experiment seems to indicate that the *"in vivo"* transformation does not take place in the fluid culture under the conditions of the assay. It is tentatively concluded that this type of genetic exchange is a consequence of the growth conditions on the agar plates.

TABLE 3

The Interruption by DNase of Genetic Recombination between Auxotrophs of Neisseria meningitidis

Selective medium used	Time when exposed to DNase	Colony forming units per ml			Recombinations per ml	
		No of units plated	No of mixed units	Frequency of mixed units	No of recombinating units	Frequency of recombinating units
Complete	–	$3\ 5 \times 10^{7}$	$8\ 7 \times 10^{5}$	$2\ 5 \times 10^{\ 2}$	–	–
A	–	"	"	"	$4\ 4 \times 10^{5}$	$1.3 \times 10^{\ 2}$
A	zero time	"	"	,	<10	$<2\ 8 \times 10^{\ 7}$
A + his	–	"	"	"	$3\ 9 \times 10^{5}$	$1\ 1 \times 10^{-2}$
A + his	zero time	"	"	"	<10	$<2\ 8 \times 10^{\ 7}$
A + pro	–	"	"	"	$3\ 7 \times 10^{5}$	$1\ 1 \times 10^{-2}$
A + pro	zero time	"	"	"	<10	$<2\ 8 \times 10^{\ 7}$
A	1 h	"	"	"	"	"
A	2 h	"	"	"	"	"
A	5 h	"	"	"	"	"
A	13 h	"	"		"	"
A	24 h	"	"	"	$3\ 0 \times 10^{2}$	$8\ 5 \times 10^{-6}$
A	48 h	"	"	"	$3\ 8 \times 10^{3}$	$1\ 1 \times 10^{-4}$

Cultures of Aux 14 thr cp⁺ and Aux 15 his pro cp were grown to middle log phase in fluid basal media appropriately supplemented Equal parts of the two cultures were mixed and incubated at 37° for 1 hour Dilutions were prepared and plated on selective media as indicated Incubation started at 37° (zero time) The [illegible] the plates Exposure to DNase was otherwise [illegible] o another, identical plate which contained a [illegible] ansformations were counted after 120 hours' incubation at [illegible] [illegible] its were determined by plate counts on blood agar plates Mixed colonies were scored by picking 400 separate colonies from the highest dilutions on blood agar plates These colonies were checked by streaking and spreading for single colony isolation (11)

In a following series of experiments the development of DNase insensitive recombinants on the surface of agar plates was investigated From the data presented in Table 3 it is seen that DNase insensitive recombinants start to appear after 13 hours of incubation After this period they gradually increase in number until a complete yield is obtained after as much as 120 hours of incubation at 37°

Since the recombination in *Neisseria meningitidis* may be considered an *"in vivo* transformation" it would be of interest to know whether genetic material passes from an incompetent to a competent cell Several experiments were performed in order to obtain some guidance concerning this problem.

The experiments recorded in Table 4 are taken as an indication that a polarity exists in the exchange of genetic information It would be

TABLE 4

Recombination between Auxotrophs of Neisseria meningitidis Indication of Polarity in Meromixis

Exper no	Competent auxotroph	Incompetent auxotroph	Selective system basal medium plus	Selected marker	Scored marker	
					Marker	Per cent of selected marker
1	his str s	orn str r	his & str	orn^+ str r	his^+	18
2	his str r	orn str s	arg & str	his^+ str-r	orn^+	100
3	his str-s	phe str r	his & str	phe^+ str r	his^+	17
4	his str r	phe str s	phe & str	his^+ str-r	phe^+	100

Cultures of the two auxotrophs were grown to the middle logarithmic growth phase in basal media appropriately supplemented Equal parts of the two suspensions were mixed and incubated at 37° for 90 minutes Serial dilutions were prepared in saline and plated on the selective plates without str After a preincubation of 6 hours for phenotypic expression of the str r marker, the plates were moved over to selective plates with 200 µg str per ml (3, 11) Colonies were picked after 3 to 4 days and scored by streaking

highly unlikely if an orn marker as well as a phe marker were found which were both 100 per cent linked with a str-r marker A more plausible hypothesis would be that the genetic material passes from the incompetent to the competent auxotroph, and that the his marker is co transformed with a frequency of 17 to 18 per cent In the system presented in Table 4 many orn str-r or phe⁻ str-r units might survive in the selective systems of experiments 1 and 3 by cross-feeding, thus being able to donate the his^+ allele in later transformations in spite of the selective system

A more suitable system for the examination of the polarity will be presented in another publication (14)

DISCUSSION

The work here described demonstrates that auxotrophs of *Neisseria meningitidis* may be transformed to prototrophy by deoxyribonucleates The frequency of transformation with nutritional markers is of the same dimension as that obtained with a streptomycin-resistance marker

An auxotroph which responds on the addition of threonine is transformed to prototrophy with a low frequency as compared to the transformation of the str-r marker Since the frequency with which double markers are simultaneously transformed proved to be far less than the frequency found if selected upon a single marker in the doubly marked auxotroph, the behaviour of the particular thr auxotroph may signify that the strain in question actually is *provided* with two different mutations in the metabolic pathway leading to thr On the other hand, the thr auxotroph is transformed to prototrophy with a higher frequency, relative to the streptomycin marker, than a simultaneous transformation of the two markers his and pro An explanation might be

during an observation time of 8 hours The experiment seems to indicate that the "*in vivo*' transformation does not take place in the fluid culture under the conditions of the assay It is tentatively concluded that this type of genetic exchange is a consequence of the growth conditions on the agar plates

TABLE 3

The Interruption by DNase of Genetic Recombination between Auxotrophs of Neisseria meningitidis

Selective medium used	Time when exposed to DNase	Colony forming units per ml			Recombinations per ml	
		No of units plated	No of mixed units	Frequency of mixed units	No of recombinating units	Frequency of recombinating units
Complete	–	3 5 × 10	8 7 × 10	2 5 × 10 2	–	
A	–				4 4 × 10	1 3 × 10
A	zero time				<10	<2 8 × 10
A + his	–				3 9 × 10	1 1 × 10
A + his	zero time				<10	<2 8 × 10
A + pro	–				3 7 × 10	1 1 × 10
A + pro	zero time				<10	<2 8 × 10
A	1 h					
A	2 h					
A	5 h					
A	13 h					
A	24 h				$3\ 0 \times 10^{2}$	$8\ 5 \times 10^{-6}$
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Cultures of Aux 14 thr cp and Aux 15 his pro cp were grown to middle log phase in fluid basal media appropriately supplemented Equal parts of the two cultures were mixed and incubated at 37° for 1 hour Dilutions were prepared and plated on selective media as indicated Incubation started at 37° (zero time) The zero time controls contained DNase in the plates Exposure to DNase was otherwise obtained by moving the plate over to another identical plate which contained a double quantity of DNase (3 11) Transformations were counted after 120 hours incubation at 37° Colony forming units were determined by plate-counts on blood agar plates Mixed colonies were scored by picking 400 separate colonies from the highest dilutions on blood agar plates These colonies were checked by streaking and spreading for single colony isolation (11)

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that the two threonine mutations are genetically linked while the his marker and the pro marker are only co-transformed

On the whole, the transformation frequencies obtained are low The numbers found, however, are reproducible Apparently the strains tested fall into two groups with regard to transformation frequency, one giving values between 10^{-2} and 10^{-3}, the other yielding frequencies between 10^{-4} and 10^{-5}

The factors which influence the yield of transformants have not been sufficiently investigated It seems, however, that a well-defined or high wave of competence is not found in *Neisseria meningitidis* cultures Instead, at any time during the first half part of the logarithmic growth phase a fairly small but constant fraction of the population is simultaneously competent The increased yield of transformations during a prolonged incubation with DNA is taken to indicate that each unit in the culture is competent only for a very short period A prolonged incubation will accordingly allow more units to pass through a transitory state of competence The problem of competence will be treated in some detail in other publications from this laboratory (12, 13)

In *Neisseria meningitidis* transformation may take place without accessory factors and by direct plating on basal agar plates A higher yield, however, is obtained with the transformation system described (2, 13)

Several markers, viz the ones concerned with growth requirements as well as with drug resistance, may obviously be simultaneously transformed by DNA in *Neisseria meningitidis* as shown above This experience raises the problem of linkage versus co transformation Many examples of true linkage in several transformation systems are now on record The determinants of streptomycin-resistance and cathomycin-resistance are closely linked in *Haemophilus* (7) The loci which determine different degrees of resistance to sulphonamide as well as the loci of str-r and mannitol fermentation seem to be linked in *Pneumococcus* (9, 10) In *Bacillus subtilis* linkage has also been demontrated between a number of different loci (17, 15, 1, 5, 6, 18)

In transformation, two loci are said to be linked if they are transformed together on the same molecule of transforming DNA When the problem was analysed according to the principles described by *Goodgal* (7), no evidence could be found of a linkage between a streptomycin-resistance marker and markers concerned with the biosynthesis of his, thr, pro and gly in *Neisseria meningitidis*

An establishment of mutual cross-feeding between nutritionally defect strains of *Neisseria meningitidis* is a regular occurrence in mixed cultures (11) This phenomenon raised several problems which are concerned with the technical approach in linkage studies The experiments performed indicate that linkage in *Neisseria meningitidis* may adequately be analysed by a technique based on scoring of single and double transformations with respect to the markers as a function of

marking DNA concentrations It is important that the observations on marker linkage between the markers studied could be unambiguously established in *Neisseria meningitidis* irrespective of the competence of the recipient culture or the transforming efficiency of the DNA preparation used (7)

So far we have considered the classical transformation of competent recipient cells exercised by DNA Another problem is the examination of an interaction of competent cells and incompetent ones when they are grown in mixed cultures

Recombinations between strains of bacteria which are competent of transformation have been described in other systems (19)

It is clear from several experiments reported in this paper that recombinations take place in mixed cultures of *Neisseria* auxotrophs provided that at least one of the participating strains is competent of transformation Since the "*in vivo* recombination is abolished when DNase is present the genetic system must at least tentatively be accepted as an "*in vivo* transformation

Several experiments were performed in order to decide the time necessary in order to obtain any DNase resistant recombinants Clearly considerable incubation time is needed from the time of plating on selective media until any recombinants start to appear With the auxotrophs used in the experiment shown in Table 3 the recombinants seem to be developed gradually in a period between 24 hours and 120 hours It was soon noted however that the total number of recombinants is very high as compared with that found in transformations with DNA In the experimental series just referred to the final recombination frequency is actually between 10^{-3} and 10^{-2} when related to the colony forming units actually plated The system is accordingly extremely effective when considered as a genetic system

This frequency of recombination must logically be considered in the light of the number of "mixed colonies found in the ... mixed cultures In previous ... carrying diff... auxotrophs ... cent of the ... 1 and 5 per ... develop mixed colonies on complete medium (11) In the present experiments the number of recombinants found correspond well with the number of mixed colonies present in the inoculate according to controls on complete media

A reasonable explanation of the "*in vivo* transformation or recombination—in *Neisseria meningitidis* would be that transformations take place within the microcolonies formed by mixed colony forming units In such colonies which are more or less immobilized on the surface of agar media the extracellular DNA might be expected to be sufficiently concentrated to transform an... 19) Also under th... to be expressed d... takes place as the... [illegible] ... cells present (1 ... have ample time ... A mating in *E coli* ... of a contact between two individual

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60
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[illegible] Regulation of chromosome replication in *Bacillus subtilis* Cold Spr Harb Symp quant Biol 28 47 54 1963
19 *Takahashi I* Genetic transformation of Bacillus subtilis by extracellular DNA *Biochem biophys Res Commun 7 467–470 1962*

cells The meromixis observed between auxotrophs of *Neisseria meningitidis* is quite different since it seems to occur as the result of a contact between populations Transformations may apparently occur in all colonies in which competent cells are present

Some experiments performed with various nutritional markers in combination with a streptomycin-resistance marker seem to support the hypothesis that a polarity exists in the meningococcal recombination system Genetic information seems to pass from an incompetent to a competent receptor cell when they occur in mixed colonies This is in agreement with the assumption that true DNA transformations take place within the colonies The problem of polarity will be treated in another publication from this laboratory (14)

SUMMARY

Auxotrophs of *Neisseria meningitidis* have been transformed by deoxyribonucleates

No genetic linkage can be found between a streptomycin-resistance marker and nutritional markers concerned with the biosynthesis of histidine, threonine, proline and glycine With saturating concentrations of DNA extensive co-transformation is found among the same markers

Genetic recombinations occur when mixed cultures of competent auxotrophs of *Neisseria meningitidis* are plated on the surface of agar media This is assumed to be due to transformation by extracellular DNA of competent cells present in mixed colonies

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Acta path et microbiol scandinav 63, 456–468, 1965

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THE INACTIVATING AND MUTAGENIC EFFECT OF ULTRAVIOLET IRRADIATION ON *NEISSERIA MENINGITIDIS*

By

Sverre Lie

Received 3 x 64

It has been the main purpose of the present paper to study the reaction of meningococci to the action of ultraviolet (UV) light

The diplococcal nature of the colony-forming unit may represent a fundamental difference between the mode of replication in this species and that of other bacteria such as *E coli*, which are more generally used in radiation studies Such a biological difference might be expected to result in a fundamentally different response to the effects of UV light The experiments accordingly were planned as a comparison between the effects of UV light on *N meningitidis* and *E coli* As a consequence of the known effects of UV light on biological systems, the investigation has to deal with two problems The first is the lethal effect of UV light, and the second the mutagenic effect

The effects registered, and particularly the differences observed between the two biological systems under investigation, should be discussed in the light of the more recent knowledge concerning the effects of UV light on bacterial systems and especially on the DNA of the cell

It has been known for a long time that the effect of UV light is greatest with wave-length around 2650 Å, close to the wave-length towards which the nucleic acids are most highly absorbent Recently it has been found that when isolated bases of the DNA are irradiated in frozen solution, irradiation of thymine leads to the formation of thymine dimers (4)

That such dimer formation might be of biological significance is indicated by the following facts Kinetic studies show that the rate of death in cells of *E coli* exposed to irradiation is correlated to the amount of thymine dimer production (27) If the thymine is replaced by the base analogue 6-azathymine (which is known not to produce dimers upon irradiation), the radiation resistance is greatly increased (28) Photoreactivating enzyme which is known to reverse the biological effects of UV irradiation in the presence of light (24) has been found to reduce the number of thymine dimers in UV irradiated DNA (31)

irradiation of transforming DNA of *H influenzae* with a wavelength of 2800 A causes an inactivation which is partly reversed by a second irradation at 2390 A, the latter having the known effect of breaking the bonds between thymine dimers (25) The same effects can be observed when the ability of denatured DNA to serve as primer for enzymic DNA synthesis *in vitro* is studied (5)

The mutagenic effect of UV light is well established, but the mode of action is still unknown The formation of thymine dimers might well be of importance as such dimers between adjacent thymines would interfere with the normal base-pairing during DNA replication If this is so one could expect a relationship between the UV light induced mutation rates and the number of adjacent thymines within the locus under investigation (5)

MATERIALS AND METHODS

Bacterial strains The strains of N *meningitidis* have previously been described (13 15) Biochemical mutants were provided by Dr *Jyssum* One required histidine for growth Aux 12 his the other required threonine, Aux 14 thre Both of these were highly transformable by meningococcal DNA A third strain employed differed from strain 12 by being incompetent in transformation by DNA (16) *E coli* as well as biochemical mutants of this strain have previously been used in this laboratory (10)

Media Heart inf sion broth (HIB and ...

... with some modifications (15) Amino acids were supplemented to a final concentration of 100 µg per ml in medium M and to 50 µg per ml in medium A Incubation of meningococci was performed in chambers containing approximately 10 per cent CO_2 In the routine technique this was achieved by adding HCl to a 5 per cent solution of $NaHCO_3$ immediately after incubation

UV treatment The source of irradiation was a Ph ...
which emits most
at a distance of 40
Samples of cells t
exposed with con
were performed un
Cells to be expo
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Bacteria grown in complete media were always spun down and resuspended in minimal media before irradiation because of the great UV absorbing activity of complete broth Cells grown in minimal media were exposed to irradiation by taking samples directly from the growth tube This could be done because mineral salts as th se employed in minimal media have no UV absorbing activity (11) Irradiation performed at 4° 22° or 37° did not alter the results "Starved" cells were obtained by grow ng ...
mented
saline ar
was con
contrast ... before and after irradiation to exclude the possibility of aggregati n ... the cells were in the form of diploc cci ... *oli* showed more than 99 per cent sing ... irradiation could be detected The r ... mutants will be described in the result section

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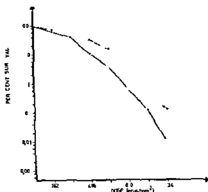


Fig 2
The UV light survival curves of *E coli* in active division (—— x —— x ——) and starvation (– o – – o – –)

Inactivation curves were also determined with cultures of *E coli* Curves obtained by using a threonine-requiring mutant of K–12, T71 are presented in Fig 2 It can be seen that although starvation increases the resistance to irradiation, the shape of the curve is not significantly altered In both cases the curve is typically sigmoidal, a frequent finding with this organism (26, 1)

The Mutagenic Effect of UV Light

The effect of UV light on the mutation rates were studied with mutations from streptomycin sensitivity (str s) to streptomycin resistance (str r) and from dependence to non dependence of a particular growth factor

The technique of isolating streptomycin resistant mutants was based on that previously described (6) Treated or untreated cells were spread on the surface of complete agar plates by means of a sterile glass rod After various hours of incubation, the agar was transferred to another agar plate of the same composition and volume, containing 200 μg of streptomycin per ml Incubation was then continued for 48 hours and resistant colonies counted In order to determine the mutation frequency, it was necessary to know the number of divisions on the first plate before transfer to the streptomycin containing medium Several authors have found a lag in the onset of division after UV irradiation, the length of the lag being in part dependant upon the intensity of irradiation (12, 8, 29) In the present experiments, the lag was determined by inoculating samples from the irradiated culture into liquid media of the same composition as the plating media (HIB) and then measuring the increase in viable cell count with time Before growth on the plates lead to crowding, it is unlikely that the growth rate should differ on these two media (8) Growth of irradiated and non-irradiated

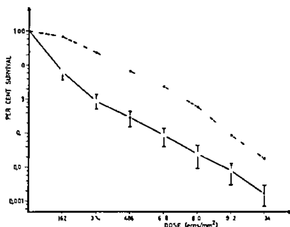


Fig 1

The UV light survival curves of N *meningitidis* in active division (—— x —— x ——), and 'starvation' (– o – o – –)

RESULTS

The Inactivating Effect of UV Light

The inactivating curves of actively dividing and "starved" meningococci are presented in Fig 1 For the actively dividing cells the curve represents the means of five experiments with the standard deviation calculated for each dose In such cultures a majority of the colony-forming units (c f u) (around 99 per cent) are apparently exponentially killed The rest, however, exhibits a much greater resistance so that a typical bipartite survival curve is obtained This type of curve was obtained with cells grown in complete medium, minimal medium and with irradiation carried out at 4°, 22° or 37° C To exclude the possibility that the bipartite shape resulted from irradiating a mixture of two genotypes differing in their radiation sensitivity, seven colonies which appeared after irradiation to a survival of 10^{5} were picked Individual survival curves determined for these strains were of the same type

One possibility for the change in slope at 1 per cent survival could be an aggregation of cells However, since the second part of the survival curve follows a straight line such an aggregation has to follow a definite pattern with the aggregates having the same sensitivity to UV light Furthermore, the inactivation curve of "starved" cells seems to indicate that the more resistant minority consists of single c f u The curve has a definite shoulder, with a subsequent inactivation rate approximately identical to that of the more resistant minority in the actively dividing culture This change in the shape of the curve is not followed by any significant decrease in viable cell count during the starvation period The greater resistance in this case can therefore not be due to the clumping of cells This is taken to indicate that the radiation resistant minority of actively dividing cells is most likely composed of single colony-forming units with the radiation resistance of "starved" cells

a survival of around 10 per cent In other experiments no increase was found at all

With the differences between the inactivation curves for actively dividing and stared meningococci in mind experiments were performed with cells in all different stages of growth grown in both complete and minimal media In no experiment UV light was found to influence to any significant extent the mutation rates to streptomycin resistance It should be emphasized that with all the three different strains used a spontaneous mutation frequency to streptomycin resistance was found which was higher than the one observed in *E coli*

TABLE 1

Mutagenic Effect of Ultraviolet Irradiation on E coli Mutation to Streptomycin Resistance (str r)

Incubation	Number of str-r colonies after irradiation to a survival of		
	100 per cent	59 8 per cent	8 6 per cent
0	0	3	1
2 hours	2	42	47
4 hours	21	50	60
No of bacteria plated	$1\ 5 \times 10^{10}$	9×10^{9}	$1\ 3 \times 10^{9}$

Each number represents the mean of five plates

TABLE 2

Mutagenic Effect of Ultraviolet Irradiation on N meningitidis Mutation to Streptomycin Resistance (str r)

Incubation	Number of str-r colonies after irradiation to a survival of		
	100 per cent	6 6 per cent	1 2 per cent
0	0	0	0
4 hours	6	0	0
6 hours	90	1	0
8 hours	*)	46	1
No of bacteria plated	9×10^{8}	6×10	$1\ 1 \times 10$

Each number represents the mean of five plates

*) Only a few resistent colonies visible due to dense background growth of sensitive cells

The mutagenic effect of UV light on the mutation from auxotrophy to prototrophy was also tested and again experiments with *E coli* served as controls The threonine-requiring mutant of K-12 T71 was grown in minimal medium supplemented with 100 μg threonine per ml medium After 24 hours of incubation the cells were spun down and resuspended in minimal medium without growth factor added Irradiated and non irradiated cells were plated on minimal agar (M) and on minimal agar supplemented with 1 per cent HIB

Table 3 shows the result of one such experiment It is evident that supplementation with broth is a necessary requirement for induced

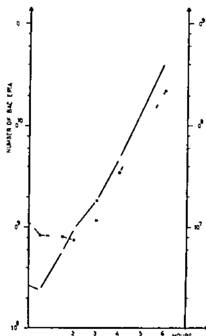


Fig. 3

Growth of UV-irradiated (– – – o – – o – –) and non irradiated (——x——x——) cells of *N. meningitidis*. Survival of irradiated cells = 6.6 per cent. Cells were transferred to broth (HIB) immediately after treatment and the growth was followed by measuring the increase in viable cell count.
Left ordinate: Number of non-irradiated colony forming units.
Right ordinate: Number of irradiated colony forming units.

meningococci in liquid media is presented in Fig. 3. A lag period of almost three hours is the result of an irradiation to a survival of 6.6 per cent. After this time the growth rate is identical to that of the control.

Similar results were obtained with *E. coli*. Irradiation to a survival of 60 per cent gave cultures that increased in number by a factor of 2 after 2 hours and by a factor of 27 after 4 hours of incubation. Irradiation to a survival of about 10 per cent gave cultures that increased in number by factors of 1.3 and 10, respectively, after the same intervals.

The results of experiments in which streptomycin served as marker are reported in Table 1 and Table 2. It is evident that while UV light is a very potent mutagen in *E. coli*, the effect on meningococci with regard to this particular mutation frequency is hardly detectable. The mutation rates obtained in these experiments can be approximately determined by measuring the increase in the number of bacteria and the increase in the number of resistant mutants during a particular time interval. This method of determining mutation rates has previously been used by *Demerec* (8). Tables 3 and 4 show the results of such analyses. With *E. coli*, the mutation rate is increased by a factor of 286.6 after irradiation to a survival of 59.4 per cent and increased by a factor of 2786 after irradiation to a survival of 8.6 per cent. With meningococci, the increase was only by a factor of around 7 after irradiation to

mutants to become expressed This phenomenon was analysed by Witkin (30) who found that it was due to the need of protein synthesis during the first hour after irradiation to ensure expression of induced mutants The number of residual divisions on the plates supplemented with broth was determined by washing off the plates with saline after 2 and 4 hours and measuring the number of viable cells With one per cent broth in the plates, the number of divisions were around five The mutation rates could then be approximately determined by dividing the number of mutants with five times the number of cells plated It can be seen from Table 5 that the mutation rate determined in this way is 710 times greater for the irradiated than for the non irradiated cells

TABLE 5

Mutagenic Effect of Ultraviolet Irradiation on E coli
Mutation from Threonine Requirement to Prototrophy

Survival	No of bacteria plated	No of prototrophs on		Mutation rate ($\times 10^{-9}$)
		M	M + 1% broth	
100 per cent	$2\ 1 \times 10^{9}$	0	2	0 19
77 per cent	$1\ 6 \times 10^{8}$	2	110	135 0

Each number represents the mean of five plates

TABLE 6

Mutagenic Effect of Ultraviolet Irradiation on N meningitidis
Mutation from Threonine Requirement to Prototrophy

Survival	No of bacteria plated	No of prototrophs on		Mutation rate ($\times 10^{-8}$)
		A	A + 1% broth	
100 per cent	$1\ 15 \times 10^{9}$	0	3	0 65
10 4 per cent	$1\ 2 \times 10^{8}$	0	3	6 2

Each number represents the mean of five plates

Similar experiments were performed with the three strains of meningococci After irradiation the bacteria were plated on minimal media supplemented with various concentrations of HIB Although spontaneous back mutants did occur in all three strains and the possibility of deletions thus will be excluded, no experiments gave a significant increase in the mutation rate as a result of UV treatment The broth content of the minimal media was varied from 0 1 to 5 per cent The number of residual divisions was approximately 4 with 1 per cent and 15 with 5 per cent broth when the inoculate was around 10^{8} bacteria Supplementation with more than 5 per cent broth gave a too high spontaneous mutation frequency due to the heavy residual growth on the plate The results from one experiment with strain 14 there are reported in Table 6 With 1 2 per cent broth, the number of residual divisions is around 5 and the mutation rates can be calculated In this particular experi-

TABLE 3

Spontaneous and UV Induced Mutation Frequencies to str-r in E coli
Analysis of Data from Table 1

Incubation	100 per cent survival			9.4 per cent survival			8.6 per cent survival		
	Number of		Rate ($\times 10^{-10}$)	Number of		Rate ($\times 10^{-10}$)	Number of		Rate ($\times 10^{-10}$)
	Bacteria	Resistant mutants		Bacteria	Resistant mutants		Bacteria	Resistant mutants	
0	1.5×10^{10}	0		9×10^{9}	3	3.3	1.3×10^{9}	1	7.7
2 hours	1.5×10^{11}	2		1.8×10^{10}	42		2.4×10^{9}	47	
Increment 0–2 hours	1.35×10^{11}	2	0.15	9×10^{9}	39	43.0	1.1×10^{9}	46	418.0
4 hours	2.16×10^{12}	21		2.5×10^{11}	50		1.3×10^{10}	60	
Increment 2–4 hours	2.03×10^{12}	19	0.09	2.4×10^{11}	8	0.33	1.1×10^{10}	13	12.0

TABLE 4

Spontaneous and UV Induced Mutation Frequencies to str-r in N meningitidis
Analysis of Data from Table 2

Incubation	100 per cent survival			6.6 per cent survival		
	Number of		Rate ($\times 10^{-10}$)	Number of		Rate ($\times 10^{-10}$)
	Bacteria	Resistant mutants		Bacteria	Resistant mutants	
0	9×10^{8}	0		6×10^{7}	0	
4 hours	1.7×10^{10}	6		1.9×10^{8}	0	
Increment 0–4 hours	1.6×10^{10}	6	3.6	1.3×10^{8}	0	
6 hours	1.5×10^{11}	90		1.5×10^{9}	1	
Increment 4–6 hours	1.3×10^{11}	84	6.2	1.3×10^{9}	1	7.6
8 hours	1.2×10^{12}	*)		1.2×10^{10}	46	
Increment 6–8 hours	1.05×10^{12}	*)		1.1×10^{10}	45	41.0

*) Only a few resistant colonies visible due to dense background growth of sensitive cells

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The National Bacteriological Laboratory Stockholm Sweden

STUDIES ON *MYCOPLASMA PNEUMONIAE* INFECTION IN SWEDEN

By

GUNNEL BIBERFELD TORSTEN JOHNSSON and JONAS JONSSON

Received 2 x 64

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pneumonia were especially selected. Later on the study was extended to include also patients with milder respiratory diseases. Altogether 239 sporadic cases of respiratory infection, hospitalized during the period July 1962–October 1963 were included in the study. Forty nine (21 per cent) of the patients were under 16 years. One hundred and seven (45 per cent) of the two hundred and thirty-nine cases had a clinical diagnosis of pneumonia and the rest had milder respiratory diseases.

SPECIMENS AND METHODS

Collection of specimens. Blood specimens were drawn from 239 patients. Throat swab specimens were collected from 149 of these cases. Blood specimens and throat swabs were as a rule taken on the first day after admission to the hospital. A second blood specimen was taken two or three weeks later. Throat swab specimens were collected in saline with 0.5 per cent bovine albumin, transported frozen in dry ice and stored at —60° C. Specimens from the nose and throat were taken also for routine bacteriological examination.

Culture medium. Agar plates were prepared with 7 parts Difco PPLO agar, 2 parts unheated horseserum and 1 part 25 per cent yeast extract as described by *Chanock et al.* (1962 c). The medium also contained penicillin (1000 units/ml, amphotericin (5 µg/ml) and thallium acetate (500 µg/ml). Broth medium was prepared in the same way except that Difco PPLO agar was replaced by PPLO broth.

Reference strain. The FH strain of M. pneumoniae kindly provided by dr. Chanock was used.

Complement fixation (CF) test. The CF antigen was prepared from the FH strain grown in broth as described by *Chanock et al.* (1962 b). Inoculated broth was incubated for 14 days at 37° C. Phenol was added to a final concentration of 0.5 per cent and the mixture was incubated for another 4 days at 37° C. The infected broth was then centrifuged for one hour at 40 000 r.p.m. in a Christ centrifuge. The pellet was resuspended in veronal buffer pH 7.4 to 1/50 of the original volume. The CF test was performed according to *Fulton & Dumbell* (1949). 4 units of antigen, 2 units of complement and sheep erythrocytes in a concentration of 0.4 per cent were used.

Fluorescent antibody technique. The procedure employed by *Chanock et al.* (1961 a 1962 a) was followed. Mycoplasma colonies from agar plates were transferred to glass slides as described by *Clark et al.* (1961), fixed in acetone and stained by the indirect fluorescent antibody technique (*Coons* 1950) using human sera diluted in phosphate buffered saline pH 7.2 and a fluorescein conjugated rabbit anti human gamma globulin serum.

Test for cold agglutinins. The procedure was similar to that described by *Smadel* (1952). 0.25 ml of a 1 per cent suspension of human erythrocytes was mixed with 0.25 ml of serial twofold serum dilutions. The serum red cell mixtures were incubated over night at 4° C.

Isolation of M. pneumoniae. Each of two agar plates was inoculated with 0.1 ml [illegible] ...ted plates were incubated at 37° C. Colonies formed [illegible] antibody technique using convalescent phase sera [illegible] infection. Each isolate was tested with two known [illegible] ...n negative human sera, all of which had been checked against the FH strain of M. pneumoniae. Colonies formed were also tested for haemolytic activity by the method described by *Somerson et al.* (1962). Agar plates with colonies were overlaid with a mixture of 5 per cent guinea pig blood in agar, incubated at 37° C and examined for haemolytic plaques after 18–24 hours.

RESULTS

Serological results. Thirty-three of 239 sporadic cases of respiratory infection showed a fourfold or greater rise in CF antibody against *M. pneumoniae*, and four patients had a CF titre equal to or greater than 1/256, although a rise in titre was not observed. Thus 37 cases showed

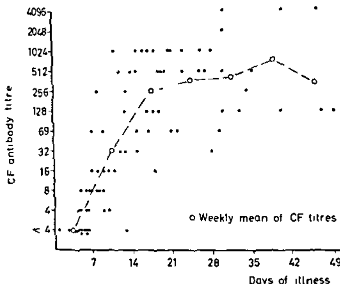


Fig 1

CF Antibody Titre Against M pneumoniae *Antigen in Relation to Duration of Illness in 37 Cases of Respiratory Disease Diagnosed Serologically as* M pneumoniae *Infection*

serologic evidence of recent infection with *M pneumoniae* Another 16 patients had CF titres 1/8–1/64 without a rise in titre The relation of antibody titre to the duration of illness in the thirty-seven cases with serologic evidence of *M pneumoniae* infection is shown in Fig 1 A significant (fourfold or greater) antibody rise was demonstrated in 20 of 20 cases with the first blood sample taken during the first week of illness, and in 11 of 15 cases with the first sample taken during the second week A significant antibody rise was found also in 2 patients with the first blood sample taken during the third week of illness All cases which had a significant antibody rise with the CF test also showed a corresponding rise with the fluorescent antibody test

Nineteen of 239 patients with respiratory infection had a significant change in cold agglutinins In 16 of the thirty-seven cases with serologic evidence of *M pneumoniae* infection a significant increase in cold agglutinins occurred In one case a significant decrease was demonstrated

Sera from the thirty-seven patients with serologically diagnosed *M pneumoniae* infection were tested for CF antibodies to adenovirus, influenzavirus A and B, parainfluenzavirus 1–4, respiratory syncytical virus and psittacosis virus Only in one case was there found a significant rise in antibody to any of the mentioned virus antigens This case had a significant antibody rise to both *influenza A* and *M pneumoniae*

Recovery of M *pneumoniae* Throat swab specimens were available from 18 of 37 cases with serologic evidence of *M pneumoniae* infection

Attempts to isolate the agent were successful in 10 of these eighteen cases Throat swab specimens from altogether 149 cases were examined The agent was not recovered from any patient who did not have serologic evidence of *M pneumoniae* infection *M pneumoniae* colonies appeared on the agar plates 6 to 9 days after inoculation All 10 isolated strains produced beta haemolytic plaques with guinea pig erythrocytes and showed specific immunofluorescence In one case *M pneumoniae* was recovered from throat swab specimens taken 18 days after the onset of illness The other 9 strains of *M pneumoniae* were isolated from throat swabs collected 4–9 days after the onset of illness

Beta haemolytic streptococci or other pathogenic bacteria were not isolated from any of the patients with *M pneumoniae* infection

Clinical aspects The age distribution of the patients in the study is shown in Table 1 *M pneumoniae* infection occurred in all age groups from 6–60 years, and was most common in older children and young adults *M pneumoniae* infections occurred during all seasons of the year

TABLE 1

Age Distribution of Patients in a Study of M pneumoniae *Infection*

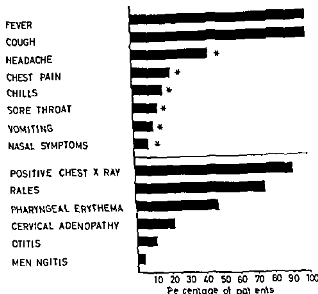
Age (years)	Cases investigated	Cases of M pneumoniae infection
0–5	14	0
6 10	13	5
11–15	22	7
16 20	20	8
21 30	19	5
31 40	19	4
41 50	19	4
51–60	29	3
61–70	40	0
71–80	34	1
81–90	10	0
Total	239	37

TABLE 2

Maximum Fever in 36 Patients with Serologically Diagnosed M pneumoniae *Infection*

Temperature	Number
37 5° 38 5° C	0
38 6° – 39 5° C	11
> 39 5° C	25

Of 37 cases with serologically diagnosed *M pneumoniae* infection, 35 had a clinical diagnosis of pneumonia and two had febrile bronchitis Otitis occurred in 4 cases and meningitis in one case Symptoms and signs are shown in Fig 2 The main symptoms were fever and cough which were present in all cases of *M pneumoniae* infection The maxi-



F g 2

Cl n cal Symptoms and S gns in 36 Pat ents w th Serolog cally D agnosed M pneumoniae Infection

mum temperature ranged from 38 6° to 40 6° C (101 5° F 105 1° F) as shown in Table 2 The average duration of fever (temperature >38 0° C) was 9 days

The roentgenological changes of the lungs were generally unilateral and located in the lower lobes Bilateral involvement occurred in 6 of 34 cases with roentgenologically verified pneumonia Apical changes were seen in 4 cases In two of these cases the changes were roentgeno log cally similar to the findings in tuberculosis

An elevated erythrocyte sedimentation rate (ESR) (Westergren) was found in all patients The maximum value ranged from 17 120 mm per hour The ESR was >50 mm per hour in 16 of 37 cases The white blood cell count was generally normal 3 patients had leucocytosis with values between 10 000 and 20 000 and 2 patients had leucopenia with a white blood cell count less than 3 000

The average duration of hospital treatment was 12 days

COMMENTS

The laboratory and clinical findings in the cases of *M pneumoniae* infection in this study are in good agreement with those described in similar investigations in other countries (USA England and Finland) (*Chanock et al* 1961 a *Cook et al* 1960 *Mufson et al* 1962 *Goodburn et al* 1963 *Jansson et al* 1964)

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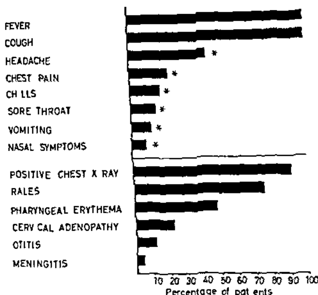


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In the study presented *M. pneumoniae* was isolated from 10 of 18 cases which were positive in the CF test. The recovery rate was lower than that reported by *Chanock et al.* (1962 c) who isolated the organism from 12 of 13 serologically positive cases with pneumonia but higher than that reported by *van der Veen & van Nunen* (1963) who isolated the organism from 8 of 25 serologically positive cases with febrile respiratory diseases.

Due to the partly retrospective nature of this study information regarding certain clinical symptoms could not always be obtained. The incidence of some of the symptoms recorded in Fig. 2 must therefore be regarded as minimum figures.

One case had evidence of double infection with *M. pneumoniae* and influenza virus A. In the other 36 positive cases no other common cause of the respiratory infection was demonstrated.

Conclusions about the frequency of M. pneumoniae infections cannot be drawn from the material studied. However, a continuous study of hospitalized cases of respiratory infection is in progress. In this study 28 per cent of cases of pneumonia investigated during the period February–June 1964 showed serologic evidence of *M. pneumoniae* infection (*Biberfeld et al.* To be published). Thus the evidence so far obtained indicates that *M. pneumoniae* is a fairly common cause of lower respiratory tract illness in Sweden.

SUMMARY

Sera from 107 cases of pneumonia and 132 cases of milder respiratory infection were examined by the CF test against *M. pneumoniae* antigen. Thirty five patients with pneumonia and 2 patients with bronchitis had serologic evidence of *M. pneumoniae* infection. All cases which had a significant antibody rise with the CF test also showed a corresponding rise with the fluorescent antibody test. *M. pneumoniae* was isolated from 10 of 18 serologically positive cases. Cold agglutinins were demonstrated in 17 of 37 cases (46 per cent) with *M. pneumoniae* infection.

M. pneumoniae infections occurred during all seasons of the year and were most common in older children and young adults. The clinical features of the cases with *M. pneumoniae* infection in this study resemble those described in similar investigations in other countries.

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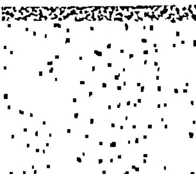


Fig 1
B1 II cells after 30 minutes in E–20 medium (20 per cent foetal bovine serum)

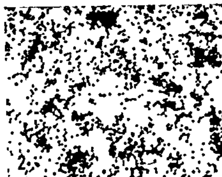


Fig 2
B1-II cells after 30 minutes in 199 solution with pronounced aggregation

Experiments 4 and 5 In Experiment 4 samples for counting were taken from a
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To A
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e was
immediately taken for counting from each tube After washing twice with Hanks BSS cell proteins were determined (*Oyama & Eagle* 1956) The cell counts and protein contents showed little spread within the B series

Experiment 5 was similar to Experiment 4 except that the B1 II cells were exposed to Versene twice as long (see Table 1) On microscope slides at room temperature droplets of the A suspension were seen to have initial and gradually increasing cell aggregation whereas the B suspension disclosed little or no aggregation. (See Figs 1 and 2)

It appears from Table 1 that samples suspended in serum free salt solution yielded lower cell counts They also showed a reduction of the cell proteins, but not of the same magnitude

Discussion and Summary

Cell

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Acta path et microbiol scandinav 63 476–477, 1965

BRIEF REPORT

EFFECT OF SERUM ON AGGREGATION OF CELLS IN ARTIFICIALLY PREPARED SUSPENSIONS

By *F Knutson P M Lundin* and *K Norrby*

In studying the viability of suspended cells we have long observed unexpected cell losses in some suspensions *Fichtelius* (1964) reported that addition of liver extract increased the absolute numl
tissue Serum was said to exert a
factor contributes to the dissolutic
ficant cell aggregation had not been demonstrated however We shall now report similar observations with mouse sarcoma cells and cultivated cells

A standardized counting procedure was used (i) thorough agitation of the test suspension, (ii) fixation of 0 2 ml with 0 010 ml 35 per cent formaldehyde, (iii) renewed agitation of the fixed sample before the transfer of cells to a Burker haemocytometer 80 B squares usually containing 100 to 800 cells were counted with the error roughly following the square root law

Experiment 1 A cell suspension produced with trypsin and DNase (*Mallen & Burl* 1961) from a transplantable mouse sarcoma MCG 1 (*Mellgren et al* in press) was used The cells were suspended in 5 ml Parker 199 Solution After counting the remainder (4 6 ml) was diluted to 10 ml with 199 solution Samples were taken from this diluted suspension Every fourth was fixed immediately and the others (1 ml each) were transferred to roller tubes containing 2 ml E 20 medium (20 per cent bovine foetal serum + 80 per cent Parker 199) From each roller tube a sample was at once fixed for counting

Cell counts disclosed that the samples without E–20 medium consistently showed a cell loss averaging 25 per cent while the counts in those samples that had been suspended in medium for some minutes were 8 per cent higher than expected
ss were separated
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oss of 60 per cent
ples suspended in
medium showed a cell loss of 6 per cent (Exp 2) and a cell gain of 12 per cent (Exp 3)

TABLE 1

	Number of tubes	Final serumconcentration in per cent	Cell number in per cent	Cell proteins in per cent
Experiment 4				
1 ml A + 2 ml 199	3	0	41	69
1 ml A + 2 ml F 20	3	13	67	75
1 ml B + 2 ml 199	2	5	100	100
1 ml B + 1 ml 199 + 1 ml E 20	2	12		
1 ml B + 2 ml E 20	2	18		
Experiment 5				
1 ml A + 1 ml 199	2	0	[illegible]	
1 ml A + 1 ml F 20	2	10	[illegible]	
1 ml B + 1 ml E 20	4	20	100	

Received 18 ii 65 *from Department I of Pathology University of Gothenburg* Sweden

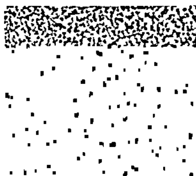


Fig 1
B1 II cells after 30 minutes in E–20 medium (20 per cent foetal bovine serum)

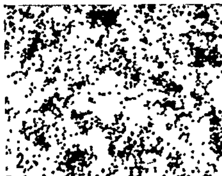


Fig 2
B1 II cells after 30 minutes in 199 solution with pronounced aggregation

Experiments 4 and 5 In Experiment 4 samples for counting were taken from a 4 5 ml suspension of B1 II cells (*Norrby et al* 1962) prepared as in Experiments 2 and 3 The suspension was then divided into two parts A and B of 2 ml each To A was added 6 ml Parker 199 and to B 6 ml E 20 medium After 5 minutes cell counts were made and A and B were further divided as shown in Table 1 A sample was immediately taken for counting from each tube After washing twice with Hanks' BSS cell proteins were determined (*Oyama & Eagle* 1956) The cell counts and protein contents showed little spread within the B series

Experiment 5 was similar to Experiment 4 except that the B1 II cells were exposed to Versene twice as long (see Table 1) On microscope slides at room temperature droplets of the A suspension were seen to have initial and gradually increasing cell aggregation whereas the B suspension disclosed little or no aggregation (See Figs 1 and 2)

It appears from Table 1 that samples suspended in serum free salt solution yielded lower cell counts They also showed a reduction of the cell proteins but not of the same magnitude

Discussion and Summary

Cell suspensions ...
cultivated cell line
with Parker 199

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References Fichtelius K E Acta path ...
Madden R F & Burk D J
(in press) —Norrby K Ert
Oyama V I & Eagle H Pr
R C Methods of Tissue Cu...

Acta path. et microbiol. scandinav. 63, 478–480, 1965

BRIEF REPORT

HISTOLOGICAL CHANGES IN THE SMALL BOWEL IN DIABETES MELLITUS

A Study of Peroral Biopsy Specimens

By *V. M. Drewes* and *Steen Olsen*

Diarrhoea and malabsorption sometimes occur in patients with diabetes mellitus, and it is believed that, in such cases, an aetiological relationship exists between the diabetes and the intestinal abnormalities.

Some authors have expressed the view that the diarrhoea is a manifestation of autonomic neuropathy. In 1958 *François & Mouriquand* claimed to have found an anatomical basis for this theory. At autopsy they revealed severe changes in Auerbach's plexus *in a young diabetic with persistent diarrhoea. However, a similar* observation had not been made before (*Berge et al.* 1956), nor has it been confirmed by subsequent investigators (*Vinnik et al.* 1962, *Bojsen-Møller et al.* 1963).

Mucosal changes similar to those observed in idiopathic steatorrhoea have been revealed in some diabetics with diarrhoea and steatorrhoea, but it has not been possible to demonstrate changes which differ from those seen in non-diabetic patients (*Green et al.* 1962, *Vinnik et al.* 1962). As far as we know, vascular changes in the small bowel have not previously been demonstrated in diabetes mellitus.

TABLE 1

Survey of Clinical and Histopathological Studies

Case no.	Sex	Age (yr.)	Duration of diabetes (yr.)	Retinopathy	Nephropathy	Diarrhoea	Steatorrhoea	Radiographic examination Small bowel	Histological examination: Kidney biopsy	Small bowel: Atrophy of mucosa	Small bowel: PAS pos. thickening of vessels
1	♀	48	11	+++	+	++(4)	++	+	++	++	++
2	♂	51	11	++	0	+(2)	+	+		0	0
3	♂	31	24	++	++	0	0	+	++	0	+
4	♂	54	26	++	++	0	+	+		0	0
5	♀	59	6	0	0	+(1)	0	0		0	0
6	♀	75	7	++	+	0	0	+		0	0
7	♂	30	17	+++	++	0		0	++	0	0
8	♀	42	13	++	++	+(7)	++	+		0	0

Retinopathy: +, micro-aneurysm; ++, haemorrhage and/or exudation; +++, proliferative changes.
Nephropathy: +, proteinuria and/or serum creatinine >1.3 mg per 100 ml; ++, proteinuria >3 g per litre and/or serum creatinine >3 mg per 100 ml.
Diarrhoea: Duration in years.
Steatorrhoea: +, faecal fat >6 g per day in periods or constantly; ++, faecal fat >20 g per day in periods or constantly.
Radiographic examination:
Small bowel: +, appearance characteristic of malabsorption syndrome.
Histological examination:
Kidney (biopsy specimen): +, diabetic changes; ++, severe diabetic changes.

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Fig 1
Arteriole in the submucosa (PAS staining)

Clinical Series Peroral intestinal biopsy by the method of *Crosby & Kugler* (1957) was attempted in 10 patients with diabetes mellitus The biopsy was successful in eight cases in which tissue samples were secured from the level of the duodeno jejunal junction

Results The most abnormal observation was made in a 48 year old woman who had had diabetes for 11 years Proliferative retinopathy and nephropathy were present and the patient had been troubled with diarrhoea for 4 years Histological examination of a biopsy specimen from the small bowel of this patient (Case 1) revealed moderately severe atrophy of the mucosa The intestinal surface was very smooth and only traces of the villous structure were present The crypts were preserved and were estimated to contain a normal number of mucous cells. Some arterioles and capillaries in submucosa showed unquestionably pathological changes in the form of pronounced strongly PAS positive usually homogeneously stained fairly uniform thickening of the walls The changes in the capillaries and arterioles were similar to those observed in other organs in long term diabetes In many vessel walls two types of substance seemed to be present one slightly fibrillar and moderately PAS positive and another perfectly homogeneous and strongly PAS positive

In another patient (Case 3)—a 31 year old man who had had diabetes from childhood the intestinal mucosa was of normal structure The submucosa and lamina muscularis mucosae revealed capillaries and arterioles with PAS positive thickening of the walls of the same nature as those seen in Case 1 but they were less severe although the histological appearance was definitely abnormal

Pronounced retinopathy and distinct nephropathy were present both in Cases 1 and 3 (Table 1)

Histological examination of the intestinal biopsy specimens from the remaining

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BRIEF REPORT

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										Atrophy of mucosa	PAS pos thickening of vessels
1	♀	48	11	+++	+	++(4)	++	+	++	++	++
2	♂	51	11	++	0	+(2)	+	+		0	0
3	♂	31	24	++	++	0	0	+	++	0	+
4	♂	54	26	++	++	0	+	+		0	0
5	♀	59	6	0	0	+(1)	0	0		0	0
6	♀	75	7	++	+	0	0	+		0	0
7	♂	30	17	+++	++	0		0	++	0	0
8	♀	42	13	++	++	+(7)	++	+		0	0

Retinopathy +, micro aneurysm, ++ haemorrhage and/or exudation +++ proliferative changes
Nephropathy +, proteinuria and/or serum creatinine >1 3 mg per 100 ml, ++ proteinuria >3 g per litre and/or serum creatinine >3 mg per 100 ml
Diarrhoea Duration in years
Steatorrhoea +, faecal fat >6 g per day in periods or constantly ++ faecal fat >20 g per day in periods or constantly
Radiographic examination
Small bowel +, appearance characteristic of malabsorption syndrome
Histological examination
Kidney (biopsy specimen) + diabetic changes ++ severe diabetic changes

[illegible] *University Clinic (Head Professor Knud*
logy (Head Professor Steen Olsen M D)
Aarhus Denmark

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The Institute of Pathology I, University of Gothenburg
(Head Professor Jan Mellgren, M D) and The Department of Pathology, Central Hospital, Vanersborg, Sweden (Head: Docent Otto Grontoft, M D)

THE PERMEABILITY TO P^{32} IN DIFFERENT REGIONS OF THE BRAIN OF NEW-BORN AND ADULT RABBITS

An Investigation of the Blood-Brain Barrier

By

Otto Gröntoft

Received 14 vi 64

It will soon be 80 years since *Ehrlich* (1885) discovered that in the central nervous the permeability to various substances differs fundamentally from that in the other organs of the body. A number of substances which can diffuse from the blood into many organs either do not penetrate into the central nervous system at all or do so only slowly An attempt has been made to explain this phenomenon by the existence of a "barrier" between the blood and the central nervous system The anatomical basis of this barrier is not known and no single anatomical structure in the brain can account for it. Investigations with trypan blue, indicate that for large molecules the barrier is connected with the capillary endothelium (*Spatz* 1934, *Broman* 1949, *Grontoft* 1954) The question whether the barrier for small molecules, such as ions, is also to be looked for in the vascular endothelium cannot be answered at present It is certain, however, that biochemical conditions play an important part in the diffusion of ions through the barrier. Excellent reviews of the whole subject were recently published by *Dobbing* (1961, 1963)

The blood-brain barrier is most simply demonstrated by means of various dyes, of which trypan blue has been most widely used because of its low toxicity If trypan blue is injected intravenously, subcutaneously, intraperitoneally or is perfused intravitally all the organs stain blue with the exception of the central nerv[illegible]
few small regions are s[illegible]
In these regions, i e pl[illegible]

The experiments in th[illegible]
at the [illegible] [illegible]ars 1955–56 [illegible]ishes to ex- [illegible]assistance in [illegible] Technology [illegible]aluabel dis- [illegible] thanks are [illegible]tal, London.

six patients showed a normal structure of the small bowel However several of them conveyed the impression that thickening of the vascular walls was occasionally present but these changes were so slight that we did not dare to attach significance to them

None of the histological sections revealed changes in the nerve cells or nerve fibres of the submucous plexus

Discussion Thus histological study of the biopsy specimens disclosed definite deviations from normal in the structure of the small vessels of the small bowel in two of eight patients with diabetes mellitus In both diabetes had been diagnosed *more than 10 years previously, distinct diabetic retinopathy and clinical nephropathy* were present and microscopic study of renal tissue had revealed severe diabetic changes Neither we nor others (*Bojsen Moller et al* 1963) have been able to demonstrate the aforementioned changes in the vessels of the small bowel in non diabetics and it must therefore be reasonable to assume that they are a manifestation of diabetic angiopathy (*Lundbæk* 1957)

A study of the small bowel from autopsied diabetics is in progress In that study we have so far found changes of exactly the same nature as those described above in the vessels of the small intestine in four out of ten patients

Summary Histological study of biopsy specimens of the small bowel disclosed PAS positive thickening of the walls of the small vessels in two out of eight patients with diabetes mellitus These changes are compatible with a diagnosis of diabetic angiopathy

Severe mucosal atrophy was demonstrated in one of the four patients with steatorrhoea

Lesions of the nerve cells or nerve fibres were not observed in any of the cases

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especially investigated in my experiments The cerebral vessels were perfused with radioactive colloidal gold (Au^{198}), which normally does not diffuse through the vessel walls (*Hevesy* 1948) By determining the concentration of this substance in different parts of the brain, relative vascularity can be estimated Furthermore the colloidal isotope Au^{198} can be determined in the same test as P^{32}

As previously mentioned the ion concentration on the two sides of the membrane is a factor that influences diffusion Therefore the amount of P^{32} has been determined both in blood and in cerebral tissue free from blood Blood was removed from the brain by means of perfusion with physiological saline solution Control of the irrigation fluid showed that the activity in it was less than 1 per cent of the activity of the blood This method of rinsing out the blood did not alter the phosphorus content of the cerebral tissue which is in agreement with *Brattgard & Lindquist*s (1955) investigation

The Donnan equilibrium is of less importance After comparing plasma and its ultra filtrate *Hastings* and co workers (1929) calculated that this factor was 0 95 for univalent ions The time factor was kept constant in all experiments

MATERIAL AND METHOD

The material consisted of a total of 90 rabbits[1] of which 50 were 24 hour old animals weighing about 60 g and 40 were adult animals weighing about 3 kg The method used was as follows P^{32} (orthophosphate) without carrier was administered intravenously in a dose of 0 4 mc to the adult animals and intracardially in a dose of 1/40 mc to the new born It is necessary that P^{32} is injected directly into the blood stream Exactly 10 minutes after injection 1 ml of blood was taken from the adult animals and ½ ml from the new borns The animals were killed 10 20 40 and 80 minutes after the injection of the isotope Immediately thereafter the brain was perfused via the aorta for 1 minute with physiological saline solution and then for 2 m [illegible] in blue and finally for 2 [illegible] mc of Au^{198} per litre Al [illegible] of in active phosphate s [illegible] ed and the soft membrane [illegible] as the regions previously referred to which normally stain with trypan blue were carefully removed After that the cerebral cortex + white matter the basal ganglia the cerebellum and the brain-stem were separated These parts of the [illegible] in 10 ml of 5 per cent trichloracetic acid T [illegible] measured twice at an interval of 6 days in [illegible] 20th century electronics) As P^{32} and Au^{19} [illegible] 2 69 days respectively—the amounts of the two isotopes can be calculated [illegible] the [illegible]

[illegible] e perfusion method are

1 The amount of P^{32} in the cerebral vessels is reduced for example in a 10 minute-experiment from 100 per cent to under 1 per cent Since the vessels form only a small part of the volume of the brain the small residue of P^{32} in the vessels is of no significance

2 The presence of an intact barrier is checked by means of trypan blue and the

[1] Only adult animals were investigated under narcosis (Numal "Roche" + aether)

hypophysis and area postrema the permeability to other substances is also higher than in the rest of the brain (*Borell & Örstrom* 1945, *Bakay* 1952) There is a blood-brain barrier to trypan blue in mammals including man, in birds, fish, and reptiles, (*Goldman* 1913, *Stern & Peurot* 1929, *Penta* 1932, *Schmid* 1934, *Broman* 1949), but this large molecule does enter the brain under pathological conditions It has also been shown that the barrier is developed and normally impermeable to trypan blue in foetal and new born humans and rabbits (*Grontoft* 1954) This has been confirmed by *Grazer & Clemente* (1957) using rat embryos

It has been suggested that there may be differences between the adult and the foetal blood-brain barrier to substances which, unlike trypan blue, normally penetrate the barrier in small quantities, such as phosphate Thus, *Bakay* (1953) found a considerably higher concentration of P^{32} in the brains of foetal and new-born than in adult rabbits Such substances might also reveal regional differences in the blood-brain barrier. *Brattgård & Lindquist* (1955), who used Br^{82} in experiments on cats, concluded that the blood-brain barrier displayed varying effectiveness in different regions of the brain

In the experimental work reported here, permeability to P^{32} in various parts of the brain was studied in rabbits and the results in new-born animals and adults were compared Phosphate ions, however, not only penetrate the blood-brain barrier, but also take part in metabolism which complicates the problem The diffusion of ions also depends on such factors as the surface area of the membrane, the concentration of the ion on the two sides of the membrane, the Donnan equilibrium and time and these factors will be dealt with first

It should be pointed out that phosphorus participates in the metabolism of the brain, *inter alia* of the myelin sheath (*Hevesy & Hahn* 1940, *Dawson & Richter* 1950, *Sperry & Waelsch* 1950) According to *Bakay* (1956) it is possible that the formation of the myelin sheaths causes the increased uptake of P^{32} in the brain, which he observed in new-born rabbits in experiments lasting for 24 hours Phosphate, however, diffuses slowly into the brain (*Hevesy & Hahn* 1946) consequently the influence of metabolism on its diffusion rate makes its self felt only in prolonged experiments In order to reduce the significance of the "metabolic factor" the experiments in this investigation were of short duration

The contact surface between the blood and the cerebral tissue consists of the walls of blood vessels The area of this contact surface depends upon the density of the vessels in the tissue and the state of contraction of the vessels

In their investigation of cats *Donning & Wolff* (1937) found that the total length of the vessels in the cortex was 2 33 times greater than in the white matter The permeable membrane is thus considerably larger in the cortex than in the white matter For this reason vascularity was

Brain stem–Cerebral cortex + white matter
$D(a_1 a_2) = 0\ 9678 \pm 0\ 17 = P < 0\ 001$
Brain stem Basal ganglia
$D(a_1 a_2) = 1\ 1508 \pm 0\ 155 = P < 0\ 001$
Cerebral cortex + white matter–Basal ganglia
$D(a_1 a_2) = 0\ 07 \pm 0\ 11$ = no demonstrable difference

TABLE 1

40 New-Born Rabbits

The Amount of P^{32} in Different Parts of the Brain Following Intracardial Injection of the Isotope The Values are given as Percentage of the P^{32}-Content of the Blood 10 Minutes after Injection

Parts of brain	P^{32}-content per g brain tissue			
	10 min	20 min	40 min	80 min
Cerebral cortex + white matter	0 958±0 139	1 263±0 195	1 750±0 282	2 270±0 308
Basal ganglia	0 925±0 127	1 150+0 168	1 660±0 290	2 210±0 303
Cerebellum	1 975±0 273	2 675±0 460	3 790±0 581	4 490±0 620
Brain stem	1 600±0 313	2 100±0 337	2 740+0 522	3 700±0 457

TABLE 2

40 Adult Rabbits

The Amount of P^{32} in Different Parts of the Brain Following Intravenous Injection The Values are given as Percentage of the P^{32} Content of the Blood 10 Minutes after Injection

Parts of brain	P^{32}-content per g brain tissue			
	10 min	20 min	40 min	80 min
Rt cerebral cortex + white mater	1 491±0 193	2 030±0 266	2 856±0 366	3 370±0 377
L cerebral cortex + white matter	1 509±0 209	2 060+0 272	2 800±0 354	3 360±0 373
Rt basal ganglia	1 073±0 146	1 545±0 195	2 222±0 294	2 640±0.319
L basal ganglia	1 064±0 135	1 356±0 206	2 113±0.316	2 460±0 285
Cerebellum	1 691±0 222	2 027±0.240	2 711±0.335	3.310±0 363
Brain stem	1 673±0 239	2 136±0 248	3 478±0 190	3 790±0 502

TABLE 3

The Amount of Au^{198} in Different Parts of the Brain in New-born and Adult Rabbits given as Percentage of the Au^{198} Content in the Perfusate

Parts of brain	New born rabbits	Adult rabbits	
	Perfusion pressure 110-125 mm	Series 1 Perfusion pressure 110-125 mm	Series 2 Perfusion pressure 135-145 mm
Cerebral cortex + white matter	1 016±0 064	1 18+0 18	7 60±0 91
Basal ganglia	1.204+0 089	1 48±0 26	9 43±1 03
Cerebellum	2 120±0 184	2 09±0 35	11 13±1 07
Brain stem	1 536±0 065	1 23+0 27	10 08±1 10
No of animals	26	12	22

regions which normally show increased permeability (plexus infundibulum area postrema, etc) are coloured blue and can readily be removed

3 Since the blood containing P^{32} has been irrigated from the vessels and replaced by the Au^{198} solution, the amount of P^{32} found in the brain must be derived from the parenchyma, and Au^{198} from the vessels

The statistical analysis has been carried out partly by a method described by *Quensel* (see *Mellgren* 1948) and partly by χ^2 analysis

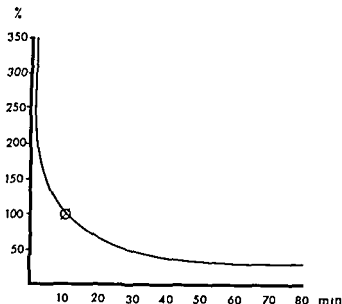


Fig 1

The amount of P^{32} in whole blood in adult rabbits during the first 80 minutes after intravenous injection, calculated as percentage of the 10 minute value

RESULTS

P^{32}-Content of Whole Blood

Fig 1 shows the P^{32}-content of the blood during the first 80 minutes after intravenous injection The curve of P^{32} falls rapidly during the first 10 minutes and thereafter relatively slowly Since the 10-minute-value of the blood is at a level that permits exact determination, the amount of P^{32} in the brain is expressed as the percentage of the blood content at this point in time

New-Born Animals

Table 1 and the upper diagram in Fig 2 show the amount of P^{32} in different parts of the brain in new-born rabbits The phosphate is calculated per *g* brain tissue free from blood The results of a statistical analysis of these curves are as follows

Cerebellum Basal ganglia
$D(a_1-a_2) = 1\ 7222 \pm 0\ 209 = P < 0\ 001$
Cerebellum Cerebral cortex + white matter
$D(a_1-a_2) = 1\ 6525 \pm 0\ 190 = P < 0\ 001$
Cerebellum Brain stem
$D(a_1-a_2) = 0\ 6896 \pm 0\ 23 = 0\ 01 > P > 0\ 001$

The amount of Au^{198} per g brain tissue is significantly different in different parts of the brain except that there is no difference between the cerebral cortex + white matter and the basal ganglia

The results of the analysis of the amount of Au^{198} and P^{32} are very similar A high or low content of Au^{198} corresponds to a respectively high or low content of P^{32} (Table 1 and 3) Since colloidal gold does not diffuse through the vascular walls it is probable that the amount of Au^{198} is due to the size of the vascular bed In this connection, it is the relative and not the absolute size which is of important Assuming that the size of the vascular bed is 1 in the cerebral cortex + white matter it will—according to Table 3—be 1 2 in the basal ganglia ,2 1 in the cerebellum and 1 5 in the brain stem In the lower diagram in Fig 2 the content of P^{32} in different parts of the brain is calculated per unit vessel by means of the values for the relative vascularity The curves are statistically analysed, but no significant differences are present Thus in the new born rabbit the deposit of P^{32} is found to be similar in all parts of the brain when calculated per unit vessel

Adult Animals

Table 2 and Fig 3 show values obtained for the P^{32} content in various parts of the brain in adult rabbits The calculation of the amount of P^{32} and the analysis of the results were carried out as for new-born animals In adult animals is was possible, however, to determine the activity for different regions of the right and the left cerebral hemispheres separately Table 2 shows that the P^{32} values for the right and the left side are in good agreement, and, consequently, in the curves in Fig 3 they have been taken together The statistical analysis of the curves (upper diagram Fig 3) of the amount of P^{32} calculated per g brain tissue gave the following results

Cerebellum Basal ganglia

$D(a_1\ a_2) = 0\ 6152 \pm 0\ 070 = P < 0\ 001$

$> 0\ 02$

$> 0\ 05$

The basal ganglia show a significantly lower amount of P^{32} than the other parts of the brain Otherwise there is no statistically significant difference between the curves

The analysis of the Au^{198}-factor was more complicated because two different perfusion pressure were used (see Table 3) In series 1, comprising 12 animals, perfusion was performed under a pressure of 110–

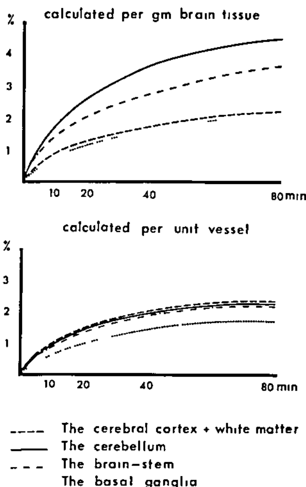


Fig 2

P^{32} content of different parts of the brain in new born rabbits calculated per g of brain tissue and—by means of the Au^{198} factor—per unit vessel There are no significant differences between the curves in the lower diagram

The amount of P^{32} per g brain tissue is significantly different in different parts of the brain, except that there is no difference between the cerebral cortex + white matter and the basal ganglia

In Table 3 the amount of Au^{198} per g brain tissue is shown The Au^{198}-solution was perfused with a pressure of 110–125 mm The analysis of the results are as follows

Cerebellum Basal ganglia
$D(a_1\ a_2) = 0\,92 \pm 0\,204 = P < 0\,001$
Cerebellum Cerebral cortex + white matter
$D(a_1\ a_2) = 1\,10 \pm 0\,195 = P < 0\,001$
Cerebellum Brain stem
$D(a_1 - a_2) = 0\,58 \pm 0\,203 = 0\,01 > P > 0\,001$
Brain stem Cerebral cortex + white matter
$D(a_1 - a_2) = 0\,52 \pm 0\,09 = P < 0\,001$
Brain stem Basal ganglia
$D(a_1 - a_2) = 0\,34 \pm 0\,128 = 0\,01 > P > 0\,001$
Cerebral cortex + white matter–Basal ganglia
$D(a_1 - a_2) = 0\,18 \pm 0\,110 =$ no significant difference

The amount of Au^{198} per g brain tissue is significantly different in different parts of the brain except that there is no difference between the cerebral cortex + white matter and the basal ganglia

The results of the analysis of the amount of Au^{198} and P^{32} are very similar A high or low content of Au^{198} corresponds to a respectively high or low content of P^{32} (Table 1 and 3) Since colloidal gold does not diffuse through the vascular walls it is probable that the amount of Au^{198} is due to the size of the vascular bed In this connection, it is the relative and not the absolute size which is of important Assuming that the size of the vascular bed is 1 in the cerebral cortex + white matter it will—according to Table 3—be 1 2 in the basal ganglia ,2 1 in the cerebellum and 1 5 in the brain stem In the lower diagram in Fig 2 the content of P^{32} in different parts of the brain is calculated per unit vessel by means of the values for the relative vascularity The curves are statistically analysed but no significant differences are present Thus in the new born rabbit the deposit of P^{32} is found to be similar in all parts of the brain when calculated per unit vessel

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Cerebellum Basal ganglia
$D(a_1\ a_2) = 0\,6152 + 0\,070 - P < 0\,001$
Cerebellum C

Cer

Pra $> 0\,02$

Bra $> 0\,0\mathrm{5}$

Cer ... rtex + white matter Basal ganglia
$D(a_1\ a\ ) = 0\,6413 + 0\,055 - P < 0\,001$

The basal ganglia show a significantly lower amount of P^{32} than the other parts of the brain Otherwise there is no statistically significant difference between the curves

The analysis of the Au^{198} factor was more complicated because two different perfusion pressure were used (see Table 3) In series 1, comprising 12 animals perfusion was performed under a pressure of 110–

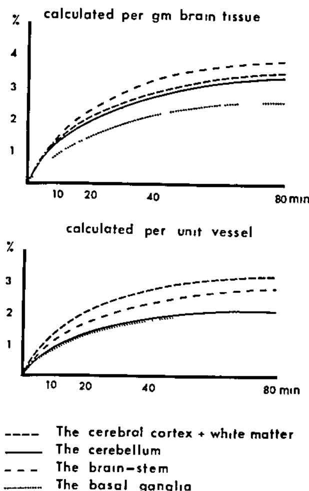


Fig 3

P^{32} content of different parts of the brain in adult rabbits, calculated per g of brain tissue, and—by means of the Au^{198} factor—per unit vessel There are no significant differences between the three lower curves—the lower diagram

125 mm, and in series 2, comprising 22 animals, the pressure was 135–145 mm The series were analysed separately and show in principle the same thing, *viz* that the amount of Au^{198} is significantly higher in the cerebellum than in the cerebral cortex + white matter In series 1 the P-value is between 0 01 and 0 001, in series 2 $P < 0\ 001$ Otherwise no statistically significant difference could be shown in the amount of Au^{198} in different parts of the brain in either series On the other hand the amount of Au^{198} in corresponding parts of the brain was higher in series 2 in which a higher perfusion pressure was used Since the blood-brain barrier had been checked with trypan blue and was apparently intact in all animals, this difference cannot be due to diffusion of the colloidal gold into the parenchyma Colloidal particles have a great propensity for adhering to surfaces A higher perfusion pressure means that a larger number of gold particles had a chance to become attached to the vessel wall in series 2 The relative values of the Au^{198}-content in

the corresponding parts of the brain in the two series are in good agreement, as is seen in the adjoining schema This would not have been the case if the Au^{198} had diffused into the parenchyma

The amount of of Au^{198}	Series 1 Perfusion pressure 110-115 mm		Series 2 Perfusion pressure 135-145 mm	
	Absolute value %	Relative value	Absolute value %	Relative value
Cerebral cortex + white matter	1 18	1 0	7 60	1 0
Basal ganglia	1 48	1 25	9 43	1 24
Cerebellum	2 09	1 77	11 13	1 46
Brain stem	1 23	1.04	10 08	1.32

In the lower diagram in Fig 3 the amount of P^{32} per unit vessel in different parts of the brain is calculated by using the relative vascularity values of series 1 The analysis of the curves showed a certain, though not fully significant difference ($0\ 02 > P > 0\ 01$) between the cerebral cortex + white matter and the cerebellum Otherwise there was no demonstrable difference between the curves

Comparison between New Born and Adult Animals

In order to study whether there is any difference in the permeability to P^{32} in the brains of new-born and adult rabbits the results of the investigations have been compared Table 3 shows that the adult animals in series 1 were perfused with the same pressure as the new-borns, which makes it possible to compare the Au^{198}-factor From this com-

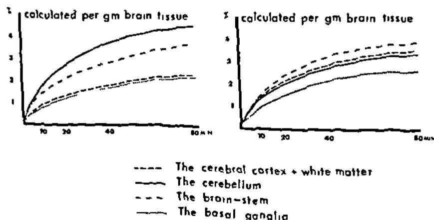


Fig 4

P^{32} content of corresponding parts of the brain in new born (left) and adult rabbits (right), calculated per g of brain tissue

parison it is evident that there is no demonstrable difference between the amounts of Au^{198} in corresponding parts of the brain Thus, since, no difference in the size of the vascular bed can be observed the amount of P^{32}, calculated per g brain tissue can be directly compared (see Fig 4) The following results were obtained in the statistical analysis

New-born rabbits show a significantly ($P < 0\ 001$) higher deposit of P^{32} *in the cerebellum than adult animals In the cerebral cortex +* white matter and in the basal ganglia of new-borns the deposit is significantly ($P < 0\ 001$) lower than in the adults In the brain-stem the same values are found in new borns and adults (no demonstrable difference)

DISCUSSION

The experiments just described show that there are certain differences in P^{32} uptake between new-born and adult rabbit brains It should be emphasised that the vascularity of the relevant parts of the brain was estimated in both groups using the colloidal isotope Au^{198} and found to be the same in corresponding parts of the brain It is, therefore, justifiable to compare the figures for the new-born and adult rabbits It was found that the uptake by the cerebellum of new born animals was higher than that of adults, whereas the uptake by the cerebral hemispheres was lower in new-borns than in adults In the brain-stem the uptake of P^{32} was equal in the two groups These differences, though statistically significant, were small, that is never more than 1 per cent These results are at variance with those of *Bakay* who found that the entry of P^{32} in the brain of the foetal and new-born rabbits was considerable higher than in the adults It is not possible, however, to compare our findings because of differences in timing and general experimental technique

Both in the new-born and adult slight but significant regional variations in P^{32} uptake were found These variations, however, are shown to depend upon regional variations in vascularity If the P^{32} uptake is calculated *per unit vessel by means of the* Au^{198} *method*, it is found to be equal in the whole foetal brain In the adult brain slight but not significant variations are present These findings are not in full agreement with *Brattgård & Lindquist* (1955), who found regional differences using Br^{82}

The time course of P^{32} uptake as well as the absolute amount found per g of brain tissue were very similar in new-born and adult rabbits (see Fig 4) It is clear, therefore, that the permeability properties of the blood-brain barrier are fully developed in the new-born animal This applies to small molecules *such as phosphate as well as to large* molecules such as trypan blue (*Grontoft* 1954, 1955 *Grazer & Clemente* 1957) The small differences which have been found may be due to metabolic factors even in these relatively short time experiments In this connection it may be of interest that the brain stem, phylogenetic-

ally the oldest part of the brain and well developed in the young animal shows the same uptake as in the adult, where as the cerebellum which grows fast at birth has a greater P^{32} uptake than in the adult

The results of this investigation thus provide further evidence that the blood brain barrier is developed in the new born and do not support the theory of a gradual maturation of the blood brain barrier

SUMMARY

The entry of phosphate, a small molecule, into the brains of new born and adult rabbits was studied using P^{32}

In order to exclude differences in P^{32} uptake due to differences in vascularity the latter was estimated using non diffusable radioactive colloidal gold (Au^{198}) and all results were calculated as P^{32} per unit vessel

Slight though significant differences in P^{32} uptake were found in new born and adult brain In the new born it was higher than adult in the cerebellum lower in the hemispheres but the same in the brain stem These differences could be accounted for by differences in metabolic activity and do not reflect a generally greater permeability of the blood brain barrier to phosphate in the young animals

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OSTEOGENIC SARCOMA ARISING IN A NON OSTEOGENIC FIBROMA OF BONE

By

JØRGEN HASTRUP and TAGE SKOV JENSEN

Received 19 ix 64

Non-osteogenic fibroma of bone was defined in 1942 by *Jaffe & Lichtenstein* (2) as a distinctive benign tumour of bone having characteristic radiological and histological appearances

Non osteogenic fibroma is encountered in children and young adults as a solitary tumour of eccentric situation in the metaphyses of the long bones X rays show a characteristic well defined multilocular translucency with a sclerosed marginal zone

Grossly the tumour presents itself as several confluent foci of fibrous tissue often of a brownish or yellowish colour

Histologically it presents a ground substance of fibromatous structure with whorls of connective tissue cells interspersed with formations of multinuclear giant cells Other areas of the tissue may be characterized by lipid filled foamy connective tissue cells The vascularity and cellularity frequently alternate from one site to another There are no signs of any formation of osteoid tissue or bony tissue but there may be remnants of a partially destroyed bone in the fibroma The presence of ample quantities of lipid containing cells in the tissue has previously given occasion to the designation xanthofibroma which has now been abandoned as the xanthoma cells are accepted as components of the non-osteogenic fibroma

Clinically a non osteogenic fibroma may give rise to symptoms such as pain and swelling but as a rule the symptoms are sparse and uncharacteristic

Non-osteogenic fibroma has been considered a benign tumour arising from the supporting tissue of the bone marrow like the so called fibrous cortical defects Following surgery in the form of curettement or resection there is no tendency to recurrence and definite cases of sarcomatous transformation have not previously been reported In 1961 however *Bel* (3) described a case of liposarcoma in the proximal end of the tibia which had possibly arisen in an originally non osteogenic fibroma In the opinion of some authors (1) this case is not sufficiently substantiated



Figs 1 a and 1 b

In the proximal end of the tibia a multilocular eccentric translucency with a sclerosed marginal zone Note the uninterrupted compact substance anteriorly on the lateral view These changes correspond accurately to those described in 1942 (cf text)

CASE REPORT

A 75 year old farmer After a mild direct injury 30–35 years ago he had noted a negligible swelling distal to the right knee This swelling slowly increased through the years but seldom bothered him in his work as active farmer Radiography in 1942 showed in the proximal end of the right tibia an area $7\frac{1}{2} \times 7\frac{1}{2} \times 8\frac{1}{2}$ cm in which the bone was expanded with a patchy marbled pattern of translucent and denser parts Its contours were somewhat irregular but well defined In 1960 there was an aggravation of symptoms Now radiography (Fig 1a 1b) showed an unchanged well defined multilocular translucency at the upper end of the tibia surrounded by a sclerosed marginal zone No biopsy specimen was obtained The patient was treated by external irradiation in an anti inflammatory dose– central dose approx 500 r

In December 1963 the patient was admitted after having had increasing local [illegible] of the right knee for a couple of months Clinical [illegible] swelling of a 7 × 8 cm area at the medial part of [illegible] e tumour which was covered by normal skin was [illegible] d areas alternating with softer parts Unlike pre [illegible] for a [illegible] viously radiography now showed a periosteal reaction as well as defective areas in the compact substance (Fig 2)

An open biopsy was done From a partially cystic readily bleeding tumour cavity a few cc of a yellowish red tissue with a few bony fragments were removed

On microscopic examination the biopsy specimen was found to con



Fig 2

(Dec 1963) On the lateral view there is now an irregular, motheaten defect corresponding to the distal part of the anterior contour

sist of several small tissue pieces, built up as a fibrillar connective tissue which in some areas was distinctly whorled and fibromatous, composed of oblong cells with regular nuclei In places, this fibromatous tissue was interspersed with some multinuclear giant cells of a somewhat varying appearance In other places there were ample quantities of large pale cells having a vacuolized, foamy, lipid filled cytoplasm



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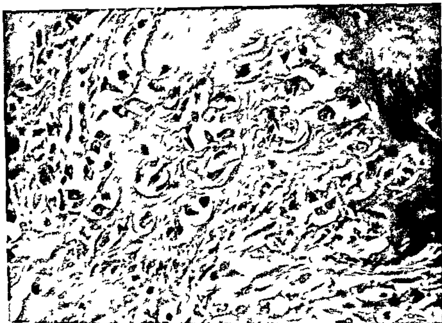


Fig 5
Part of lymph node metastasis (1963) Sarcomatous tissue with formation of osteoid partially calcified tissue

and entirely regular, small, dark nuclei (Fig 3) The vascularity varied from site to site From this tissue, which was entirely characteristic of non osteogenic fibroma, there was a transition to a more cellular tissue with pronounced polymorphism of densely arranged, plump, abnormal connective-tissue cells with hyperchromatic, irregularly shaped nuclei having conspicuous nucleoli In these areas there were numerous mitoses, in places an intercellular substance of osteoid nature, but nowhere any signs of bone formation (Fig 4)

Histologically, the lesion was characterized as a non osteogenic fibroma with transition into sarcoma

The histological impression of malignancy was confirmed when an enlarged, firm lymph node was removed from the right groin This lymph node was completely infiltrated with sarcomatous tissue in which fibrillar areas alternated with areas consisting of giant cells, osteoid tissue, and regular bone formation (Fig 5) This metastatic structure was characteristic of an osteogenic sarcoma

Chest radiography revealed uninvolved lung fields, and films of the skeleton showed no abnormalities There were no signs of hyperparathyroidism, as serum calcium, serum phosphorus, and alkaline phosphatases were normal

After palliative tele-cobalt therapy (central dose 4,800 r/26 days) to



Figs 3–4

Fig 3 Part of biopsy 1963 Fibromatous tissue with a number of large xanthoma cells

Fig 4 Part of biopsy from 1963 Cellular sarcomatous tissue with numerous mitoses



Fig 5
Part of lymph node metastasis (1963) Sarcomatous tissue with formation of osteoid partially calcified tissue

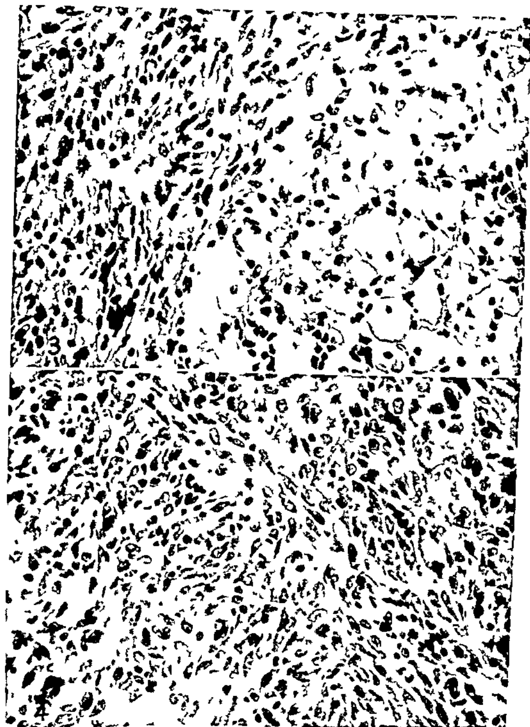
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Figs 3 4

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No such case has previously been reported The differential diagnosis is discussed

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3 *Retz L D* Primary liposarcoma of bone J Bone Jt Surg 43 123 129 1961 a

the right knee joint the pain disappeared and the patient could again stand on his right leg

DISCUSSION

The history, the characteristic radiographic findings, and the characteristic microscopic appearances in parts of the biopsy from 1963 must justify a classification of the long-standing bone tumour as a non-osteogenic fibroma from which an osteogenic sarcoma had arisen and given rise to lymph-node metastases

From a differential diagnostic point of view, the tumour is easily distinguishable from the giant-cell tumour (osteoclastoma) which, if untreated, shows a more blurred radiological appearance, not demarcated by a sclerosed marginal zone Microscopically, there was a distinct difference between the typical fibromatous tissue in the present tumour and the more cellular tissue, predominated by numerous giant cells, in an osteoclastoma

Against tumours such as a benign osteoblastoma and osteoid osteoma, the present tumour may easily be differentiated by the absence of osteoid and osseous tissue

An aneurysmal bone cyst often manifests itself as an osteolytic, well-defined tumour situated in the metaphysis of the long bones The extremely ample vascularity in the fibrous tissue, which is often the seat of giant-cell reaction and reparative bone formation, is a significant differential diagnostic sign against non-osteogenic fibroma

Histologically, the non-osteogenic fibroma is not reminiscent of fibrous dysplasia, which in a monostotic form may be of the same localization Simple bone cysts, chondromyxoid fibroma, and a monostotic form of Paget's disease are easy to rule out histologically

CONCLUSION

It has been confirmed that a malignant tumour of bone developed at a site where a benign tumour had been present for 20 years

estage represents

findings in 1942

and 1960 were characteristic, 2) on microscopic examination in 1963 large areas of the tumour were found to be of an architecture like a non-osteogenic fibroma

SUMMARY

For 30–35 years a 75-year-old man had been suffering from swelling and tenderness of the proximal end of the right tibia Since 1942 there had been constant radiological changes of the bone of a type like those seen in non-osteogenic fibroma A biopsy in 1963, occasioned by an aggravation of the symptoms, showed a histologically typical non-osteogenic fibroma, with transition in places into sarcoma Moreover, a

regional lymph node metastasis was found to be built up as an osteogenic sarcoma

No such case has previously been reported The differential diagnosis is discussed

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CYSTIC DISEASE OF THE KIDNEYS

A Microdissection Study

By

O HEGGO[1] and J B NATVIG

Received 2 viii 64

An extensive literature has accumulated concerning the peculiar and not infrequent condition of cystic disease of the kidneys However, the literature contains much speculation as to the morphological changes, the pathogenesis and the aetiology, and many hypotheses with no clear basis in observation have been advanced On the other hand, proven observations are very often adapted to some preconceived theory The differences in opinion are to a great extent due to difficulties in distinguishing cysts in the nephrons from those in the collecting tubules, probably because of the limitations of the ordinary techniques of studying conventional histological sections and preparing reconstructions from serial sections

Theories as to the development of the kidney have played a great rôle in many of the hypotheses concerning the morphology, the patho genesis and the aetiology of the cyst formation It is now well established (*Schreiner* (1), *Patten* (2), *Hamilton, Boyd & Mossman* (3)) that the metanephros originates from two separate primordia, the metanephric diverticulum of the mesonephric duct and the metane phrogenic tissue, forming the excretory system and the nephrons respectively

It is important to remember that as the pelvis and calyces expand, the straight collecting tubules belonging to the earlier orders degene rate, and so do the corresponding nephrons Hence in the fully formed kidney it is the tubules of the fifth order that open into the minor calyces

Two theories of cyst formation have been derived from the embryo logical history

1 Attempts to establish proper union between the two separate components have failed The cysts presumably arise from functional but closed nephrons (*Hildebrand* (4), *Nicholson* (5), *Langman* (6))

---

1 The microdissections were performed by O H

2 Nephrons detached from the *first orders* of collecting tubules fail to degenerate and instead become progressively enlarging cysts (*Kampmeier* (7)) *Norris & Herman* (8) suggest that in the case of cystic kidneys a much greater part of the metanephros is "provisional", and that the nephrons not only from the first orders of the collecting tubules fail to degenerate

However, there are disagreements on both these developmental theories *Allan* (9) states that it does not appear that the two portions grow separately with final union of separate lumens The collecting tubules ramify and use the metanephrogenic cells to bild the tubular and glomerular extensions directly and progressively *Oliver* (10) by dissection of developing nephrons also found that these were never at any time separated by any distance from the collecting tubules, which induced their development Cysts, he states, cannot therefore arise from a failure on the part of the growing tubules to establish the necessary contact with the collecting system Further he finds that at no time after the 3rd month do nephrons degenerate or disappear, nor do they become dilated and then make new connection with the collecting system Degeneration as demonstrated by *Kampmeier* (7), occurs only in those nephrons which form the 4th or 5th primary orders of the collecting tubules during the first month These early collecting tubules and the tissue around them disappear completely as they are taken up into the pelvis and consequently there are no potential sources of cyst formation on this basis

One of the types of cystic disease of the kidneys, which occurs in the pre and neonatal period, is that known in the literature as Potter's hamartomatous variety (*Potter* (11), *Heggo & Natvig* (12) This type is dealt with in the present paper The problems to be investigated have been

1 In which parts of the kidney parenchyma do the cystic formations occur?
2 Are other abnormal conditions manifest in any part of the urinary system in these kidneys?
3 Is it possible to say anything about the pathogenesis of the cyst formation?

MATERIAL AND METHODS

The material consists of kidneys from four girls and one boy with identical changes in the kidneys and livers Two parts were siblings All died between 2 and 31 hours after birth [illegible] ere subjected to microdissection [illegible] wed that the cystic kidneys were [illegible] of these 3 cases urine was noted

[illegible] with reconstructions of cysts from serial sections were performed in one case However this method was abandoned because it was found impractical for three reasons 1 It was time-consuming 2 It was not always possible to be con

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The University of Bergen School of Medicine, The Gade Institute, Department of Pathology (Head Professor F Waaler, M D), and Institute of Anatomy (Head Professor W Harkmark, M D), Bergen, Norway

CYSTIC DISEASE OF THE KIDNEYS

A Microdissection Study

By

O HEGGO[1] and J B NATVIG

Received 2 viii 64

An extensive literature has accumulated concerning the peculiar and not infrequent condition of cystic disease of the kidneys However, the literature contains much speculation as to the morphological changes, the pathogenesis and the aetiology, and many hypotheses with no clear basis in observation have been advanced On the other hand, proven observations are very often adapted to some preconceived theory The differences in opinion are to a great extent due to difficulties in distinguishing cysts in the nephrons from those in the collecting tubules, probably because of the limitations of the ordinary techniques of studying conventional histological sections and preparing reconstructions from serial sections

Theories as to the development of the kidney have played a great rôle in many of the hypotheses concerning the morphology, the pathogenesis and the aetiology of the cyst formation It is now well established (*Schreiner* (1), *Patten* (2), *Hamilton, Boyd & Mossman* (3)) that the metanephros originates from two separate primordia, the metanephric diverticulum of the mesonephric duct and the metanephrogenic tissue, forming the excretory system and the nephrons respectively

It is important to remember that as the pelvis and calyces expand, the straight collecting tubules belonging to the earlier orders degenerate, and so do the corresponding nephrons Hence in the fully formed kidney it is the tubules of the fifth order that open into the minor calyces

Two theories of cyst formation have been derived from the embryological history

1 Attempts to establish proper union between the two separate components have failed The cysts presumably arise from functional but closed nephrons (*Hildebrand* (4), *Nicholson* (5), *Langman* (6))

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1 The microdissections were performed by O H



Fig 2

Thin walled cysts two of which are fused together and communicate through wide openings No nephrone are connected with the tips of the cysts Unstained preparation × 65



Fig 3

impression of hyperplastic tubular epthelium Unstained preparation × 33

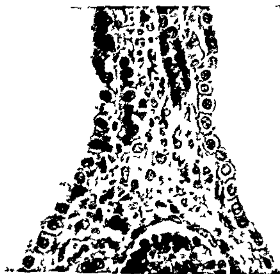


Fig 4

Ph t micrograph sl owing parts of cyst walls with a high cuboidal epithelium Hematoxylin and eosin stain × 560



Fig 1

Cut surface of a kidney showing more or less radially arranged cysts in the medulla and cortex Note the conglomerates of tortuous labyrinthic cavities deep in the medullary region † × 3 5

vinced of the continuity of the structures under reconstruction 3 Lastly and most important one obtained only a few models which might not even be representative

Stimulated by the works of *Oliver* (10 13) microdissection was performed to study the problems of the cystic kidneys

Blocks of formalin fixed kidneys were mascerated in 25 per cent HCl at room temperature according to an old method (*Peter* (14) *Oliver MacDowell & Tracy* (15)) A suitable thickness of the tissue pieces was found to be 3 mm and the length along the capsule about 10 mm The whole width of the kidney from capsule to papillae was used After masceration the blocks were washed many times in water and then stored in water of room temperature for from 12 to 24 hours In this way identification and dissection was easier possibly because of a little swelling of the structures especially the nephrons The pieces were dissected under a binocular stereoscopic microscope at a magnification of 25 or 40 times Fairly complete dissection was carried out Photographs of the material unstained and still floating in the water were taken

PRESENTATION OF THE MATERIAL

The Collecting System

Most of the cystic cavities were arranged more or less radially in the medulla and cortex (Fig 1) In the more extreme examples they practically replaced all the renal structures in these regions, although some persisting nephrons could be seen The walls of the cysts varied in thickness, even within the same sample of kidney tissue Some of the cysts were comparatively thin walled and translucent, and they were often fused together with an anastomosis between them (Fig 2) These



Fig 7
Part of a papilla with openings some of which are distinctly dilated × 35

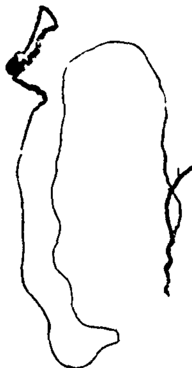


Fig 8
One of the most normal looking nephrons studied However the proximal convolution is not quite well developed and instead of a distal convolution there is a long narrow portion of a tubule The collecting tubule seems almost normal
Unstained preparation × 33



Fig 6

Cut surface of a papilla showing two papillary ducts opening into the pelvis, both draining a dilated straight collecting tubule × 35



Fig 5

Two joined cysts follow a series of linear cysts in the previous generation of collecting tubules Between the linear cysts there is an interval of undilated tubule Unstained preparation × 33

cysts were lined by a single layer of very low cuboidal cells It did not prove possible to follow such cysts far enough down the medulla to search for a possible atresia However, the majority of the cysts had a greater wall thickness (Fig 3), which was apparant even on microdissection Microscopically, they were likewise lined by a single layer of epithelium, but the cells were higher cuboidal (Fig 4)

All the cystic cavities seemed to be derived from the collecting tubules The last generations of tubules were as a rule diffusely enlarged,





Fig 11 Fig 12

Fig 11 Part of a cyst with 3 nephrons of a tortuous appearance, not unlike metanephric tubules in early development Unstained preparation × 65
Fig 12 Cyst with particularly thin proximal tubules, the one tubule possibly disrupted during dissection Unstained preparation × 33

thing obviously abnormal was found in the shape of the calyces and pelvis

Interspersed with the cysts were groups of normal collecting tubules or tubules with only slightly abnormal features (Fig 8)

The number of generations seemed to be normal

There was no increase in connective tissue around the cystic tubules or elsewhere in the kidney

The Nephrons

The total number of nephrons connected to each collecting tubule was normal The renal corpuscles were normal in size and shape. Dilatation of Bowman's capsules was never found Many nephrons, those attached to normal collecting tubules, had a nearly normal appearance



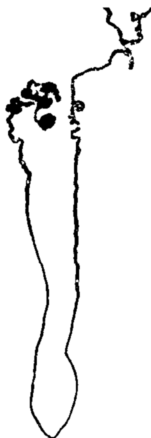


Fig 9 *Fig 10*

Fig 9 Dilated or cystic distal convolutions and minute cyst in the loop of Henle (on the right) Unstained preparation × 33

Fig 10 Normal nephron for comparation, dissected from a kidney of a premature infant total weight 2500 grams The renal corpuscle was located in the middle of the cortex Unstained preparation × 33

the earlier very often saccular Between the saccular cavities were intervals of an undilated tubule (Fig 5) There were connection between the cystic collecting tubules and the pelvis

In the deeper layers of the medulla a great number of cysts had a very tortuous, labyrinthic appearance These cysts occupied considerable areas of the kidney (Fig 1) Conglomerates of such tortuous structures were found, and between the cavities were numerous communications through wide openings It was impossible to isolate the individual units, because of the extended connections between them Furthermore, the continuity upwards was often uncertain On the other hand the communication with the pelvis was formed by radially arranged tubules, some of them more or less dilated, others narrow (Fig 6) On each papilla were 10 to 18 openings Many of these were abnormally dilated, and so were the corresponding papillary ducts (Fig 7) No-



Fig 11



Fig 12

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However, some of them showed poorly developed convolutions, especially the distal convolution, which were often absent (Fig 8)

The majority of the nephrons were markedly abnormal The tubules were short, and the convolutions, both the proximal and the distal, as well as the loop of Henle were as a rule poorly developed or even absent (Fig 5) In comparatively few nephrons the angle of the loop of Henle was the site of minute cysts, or the distal convolution was slightly dilated or cystic (Fig 9) The nephrons with renal corpuscles nearer to the surface of the kidney were invariably the most abnormal ones (*Fig 5 and 9*) *Some of these had a tortuous appearance, not un*like metanephric tubules in early development (Fig 11) The tubules belonging to renal corpuscles deeper in the cortex regularly showed a more mature picture, in that a loop could be identified, and likewise one or both convolutions, most often the proximal convolution (Fig 3) However, every portion of the nephron was considerably shorter than normal

There were no convincing disruptions of proximal tubules, with the exception that perhaps some of the thin-walled cysts, to which no nephrons were connected, might have had some at earlier stages

Fig 12 shows particularly thin and possibly atrophic proximal tubules, on dissection some doubt arose about the continuity in one place, though it surely must have existed

There were no signs of atresia, and the nephrons opened, some widely, into the cysts

DISCUSSION

The pathological alterations of the kidneys described here are of the so-called Potter's hamartomatous variety of cystic kidney disease, which is characterized by the following criteria (*Potter* (11)) The kidneys are symmetrically enlarged Close inspection reveals the entire surface to be composed of minute cysts 2–3 mm in diameter On sectioning these are found to be the ends of dilated tubules whose long *diameters are at right angles to the external surface* Varying parts of the cortex and all of the medulla are composed of greatly enlarged tubules lined by a single layer of cuboidal cells On microscopic examination varying numbers of normal glomeruli surrounded by normal Bowman's capsules and attached to normally appearing tubules can be demonstrated immediately under the capsule of the kidney The amount of connective tissue is not increased Cystic hyperplasia of the bile-ducts in the liver was present in Potter's as well as in the present cases *Oliver* (10) also states *that this variety is a specific type* However, just what the term means, or why it is applied to this variety, does not seem to be clear

It contrasts with another type, described by *Oliver* (10, 13) and named microcystic renal disease or microcystic kidney The latter condition also occurs in the pre and neonatal period and the cysts are

mostly located in the *nephrons*, the Bowman's capsules and tubules The cavities are minute, visible with a hand lens and sometimes to the unaided eye In the least involved cases the kidney may pass as normal even after a thorough microscopic examination The structural alterations are described as a dysplasia, which involves the nephrons and results in atresia and segmentation of the tubules and atrophy, sometimes with disruption and ultimate destruction of nephrons Apparently secondary to these alterations there is a cystic dilatation and a compensatory *hyperplasia* in lesser involved nephrons or parts of the individual affected nephrons The collecting system is relatively little affected, and the changes which are apparent can be explained as secondary effects due to the architectural disarrangment of the abnormal nephrons Certain cases of "infantile nephrosis" are found to be connected with this type of cystic disease (*Oliver* (13), *Fetterman & Feldman* (16)) According to *Oliver* (10) this condition cannot lead to the subsequent development of the enlarged "polycystic" kidney of adult life because of its regressive nature

The most striking difference between these two types of cystic alterations which are both found in the pre- and neonatal period is the localization of the cysts In the variety investigated here, the cavities are confined to the *collecting system* Some of them are very thin walled It has not proved possible to exclude that obstruction may play a part in the formation of these cysts for instance by congenital atresia On the other hand disruption of expanding cysts may well give rise to fusion and it is not impossible that stenosis may perhaps come about in this way As to the conglomerate of tortuous structures described in the deeper portions of mellula, where it as a rule was quite impossible to isolate the different cysts obstruction on this basis could not be excluded The tubular epithelium in the thin walled cysts is flattened cuboidal and at the same time increased in amount

However *most of the cysts* are comparatively thick walled the epithelium being high *cuboidal* and apparently much increased There is connection between such cysts and the pelvis The number of pori is normal and so is the shape of the calyces and pelvis

Some of the nephrons are well developed, but the great majority are short and in many the various segments, the thick proximal convolution loop and distal convolution can only be recognized indistinctly The nephrons may be described as primitive or poorly developed ... a f... [illegible]

sc... [illegible]

which were probably [illegible]
the nephrons in the [illegible]
served

Thus the disturbances in development seem to be different in these two types of cystic disease of the kidney

As to the pathogenesis none of the two main theories of cyst formation can be valid However, another theory, which has been eclipsed by the others, is of much interest in this connection That is the so-called neoplastic theory introduced by *Brigidi & Severi* (17), who regarded the cyst formation as due to an epithelial outgrowth The theory was supported on different premises by *Nauwerck & Hufschmied* (18), *v Kahlden* (19), *Borst* (20) and *Berner* (21) *Bunting* (22) stated that the cysts were due to abnormal proliferation of epithelium in ducts which otherwise appeared normal Numerous mitotic figures in the cells lining the cysts gave support to the hyperplasia theory, the presence of an increased number of cells being in all probability due to some abnormal stimulus

The pathogenesis of this sort of cystic kidney seems to be as follows An irregular distributed hyperplastic proliferation of the epithelium has occured in the ducts of the collecting system with the production of more or less radially arranged cysts up to the point where both cortex and medulla are filled with these cystic cavities Whether or not obstruction plays a part in their distension, is not certain, but it is not impossible However, there seems to have been an oversimplification in the attempt to explain the cyst as a dilated tubule resulting from such obstruction As for example in the cyst formation shown in Fig 5, it might be difficult to conceive of such a pattern developing purely as the result of passive dilatation Connection can be demonstrated between cysts and the pelvis

The changes in the nephrons are principally those of a dysplasia, which can be explained as secondary to the changes in the collecting system, because of a disturbance of the normal inductive process on which the development of the nephrons depends The possible atrophy of comparatively few nephrons may be due to simple pressure effects and to overstreching or disturbances of circulation because of the expanding cysts In spite of this the nephrons have mostly maintained continuity with the cysts

According to the pathogenesis, put forward above, the old question of continuity between nephrons, cysts and pelvis seems to become academic There is without doubt continuity from renal corpuscles to pelvis in some instances, in others there may not be Of greater interest is to know how many nephrons and collecting ducts are patent An answer could be given by a count, which would be excessively laborious The best answer to such a question seems to be if there is any urine in the pelvis or bladder or not In one of the cases investigated here, urine was noted in the bladder On the other hand, it is hard to believe that a kidney composed of the nephrons presented in these cases is of much functional value

SUMMARY AND CONCLUSIONS

Cystic kidneys from 5 newborn infants were investigated The kidneys from 3 cases were subjected to microdissection In all these cases the pathological alterations could be grouped under the so-called Potter's hamartomatous variety, occurring in the pre and early neonatal period The following conclusions were drawn

1 The cyst formations are derived from the collecting system
2 Most nephrons reveal extensive changes mainly in the form of a dysplasia The nephrons show continuity with the collecting tubules
3 The primary disturbances are those in the collecting system, expressed as a hyperplastic proliferation of the tubular epithelium On the other hand obstruction may play a part in the distension of some cysts The alterations in the nephrons are secondary and can be explained as due to abnormities of the inductive process, and possibly as atrophy due to mechanical effects

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AN ANGIOGRAPHIC STUDY OF CADMIUM INDUCED VASCULAR LESIONS IN THE TESTIS AND EPIDIDYMIS OF THE RAT

By

MIKKO NIEMI and MARTTI KORMANO[1]

Received 3 x 64

The general histological changes in the testis induced by the administration of cadmium are well documented in several recent papers (*Parizek* 1957, 1960, *Kar & Das* 1960, *Mason et al* 1963) However, little information concerning the mechanism of this injury is available although evidence has been accumulating to suggest that the testicular necrosis is primarily caused by interference of cadmium with the testicular blood supply (*Kar & Das* 1960, *Gunn et al* 1963) The arguments in favour of a primary circulatory disturbance are, however, mainly indirect, since changes in the testicular vascular bed has been directly demonstrated only by *Chiquoine* (1964)

Recently angiographic techniques have been successfully applied to studies of pathological changes in the vascularization of other organs e g the kidney (*Ljungqvist & Lagergren* 1962) and they have also been used to describe the normal vascular pattern of the testis (*Harrison* 1951) It was therefore decided to investigate the acute effects of cadmium on the testis circulation by an angiographic approach This report will also include an account of the late re establishment of new blood vessels to the testis Finally, the results of an application of high resolution microangiography to the testis will be given

MATERIAL AND METHODS

Adult male rats of the Sprague Dawley strain were used The age of the animals varied from 4 to 9 months For the preparation of the [illegible] were used 19 of these were [illegible] chloride (0 03 mM/kg) and 8 [illegible] killed 6 14 16 24 36 38 47 66 [illegible] the injection

The microangiographic procedure was as follows after decapitation the thorax was opened and the aorta canulated An intra arterial injection of 5000 I U heparin was given Barium sulphate ("Micropaque") was now infused for 1 to 4 hours as a fine suspension in water at 20° C (*Ljungqvist & Lagergren* 1962) The perfusion

[1] This study has been supported by a grant from *P O Klingendahl Foundation* to Dr Kormano

pressure was sometimes gradually risen up to 150 mm of mercury although most of the infusions were carried out at a pressure of 100 mmHg However even the higher injection pressure did not cause any apparent capillary leakage in control animals The control animals were perfused with the contrast medium in pairs with experimental animals using the same pressure source for both

After completion of the barium sulphate injection, the blood vessels of the testis were ligated and the testis and epididymis extirpated en block The radiographs were prepared on a fine grain X ray emulsion (Kodak's "Microtex") The tissue was then fixed in 10 per cent formalin for several days dehydrated and embedded in paraffin wax Slices were cut at 500 and 1000 µ exposed in contact with a high resolution emulsion (Kodak's 'Maximum Resolution Plate') using a Siemens X ray tube equipped with a copper anode and a beryllium window The focus to film distance was 30 cm and the tube voltage used was 20 kV

Another series of 6 rats were given a slow (2 to 3 min) intravenous injection of 5 ml of 25 per cent thorium dioxide ('Thorotrast) under aether anaesthesia This was done 0 3 and 6 hours after they had been injected with cadmium chloride similarly as the animals in the former series The testicular blood vessels were ligated immediately after the administration of Thorotrast, the testis and epididymis removed and subsequently radiographed on a fine-grain emulsion

RESULTS

1 *Angiography*

Normal vasculature of the rat testis At both 100 and 150 mmHg perfusion pressure a satisfactory filling of the testicular vascular bed was produced (Fig 1) However, the spermatic artery as well as its main branch, the testicular artery, could not be seen because of filling of the surrounding pampiniform plexus The internal testicular artery with its branches was, on the contrary, well visible as far as the resolution of the radiographic technique permitted The vasal artery was likewise well visible and its anastomosis with the testicular artery could be localized The venules of the testis were also visible, and they could be seen to form a *venous sinus by confluence at the hilus of the testis*

Effect of cadmium injection The changes observed in the testicular vasculature were few and inconstant 6 hours after the cadmium administration A decrease in the number of fine branches of the intratesticular artery was, however, sometimes seen 14 hours after injection,

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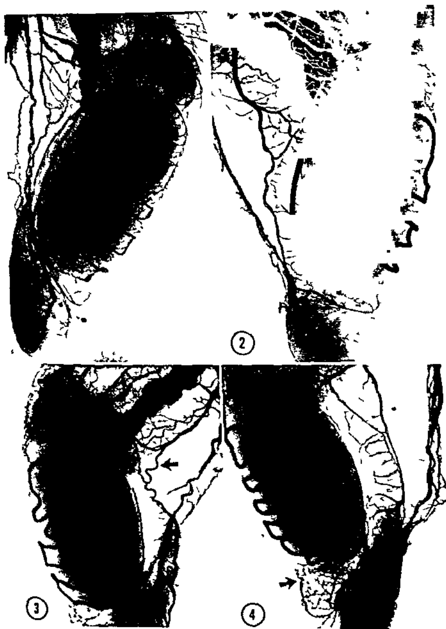
Figs 1 4

Fig 1 The vascular pattern in a normal rat testis and epididymis The vasculature has been infused with barium sulfate solution at a pressure not exceeding 150 mHg for 3 hours The pampiniform plexus is well filled obscuring both the spermatic artery and testicular artery × 4

Fig 2 Angiogram taken 14 hours after a single subcutaneous injection of cadmium chloride The small vessels of the testis are diminished in number and the veins are less filled than in the control testis × 4

Fig 3 24 hours after the cadmium injection The sparseness of the intratesticular blood vessels is evident and the anastomotic vessels between the internal [illegible] lar artery and the vasal artery (arrow) is prominent The pampini [illegible] dium × 4

Fig 4 [illegible] intratesticular vessels [illegible] rrow) appearing be [illegible] en the cauda epidid [illegible]


2
3
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Fig 3 24 *hours* after the cadmium injection The sparseness of the intratesticular blood vessels is evident and the anastomotic vessels between the internal testicular artery and the vasal artery (arrow) is prominent The pampiniform plexus is incompletely filled with the contrast medium × 4

Fig 4 36 hours after the cadmium injection Almost all of the intratesticular vessels have remained unfilled Note the capillary network (arrow) appearing between the cauda epididymis and the testis × 4

the number of visible intratesticular arterial branches was obviously decreased (Fig 2) Filling of the testicular veins with the contrast medium was negligible The vascular bed of the caput epididymis was extensively filled At 24 hours after the treatment, a conspicious absence of small intratesticular arteries was observed (Fig 3) The venous return of the contrast medium was almost completely obstructed making visualization of both the testicular and spermatic artery possible The anastomosis between the testicular and vasal arteries was readily visible 2 to 3 days after cadmium injection the internal testicular artery was completely blocked (Figs 4, 5 and 6) The venous return was likewise arrested but some anastomotic retrograde venous filling seemed to occur in the hilar area (Fig 4)

The vascular bed of the caput epididymis became more dense and coarse up to 66 hours after the cadmium administration Later on, extravasation of the contrast medium was observed (Fig 6) The cauda epididymis was virtually unaffected within the first 66 hours After 24 hours, a new network of small vessels made its appearance in the area between the cauda epididymis and the lower pole of the testis A similar network of new vessels was seen in the caput epididymis

After a time lapse of 5 days or more from the cadmium exposure the anastomosis between the internal testicular and vasal arteries was poorly filled with the contrast medium (Fig 5) At the 11th day, leakage of contrast medium to the testis parenchyme was always noticed (Fig 7) even at an infusion pressure of 100 mmHg The vascular bed of the cauda epididymis gradually became disorientated, the capillary loops forming coarse bundles instead of a fine network (Fig 6)

One month after the cadmium administration a reappearance of new vasculature in the testis was evident (Fig 8) The course and distribution of the new blood vessels did not, however, correspond to those of the original testicular vessels At this stage, the testis was maximally involuted

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Figs 5–8

Fig 5 66 hours after the cadmium injection The te[illegible] tain a single blood vessel [illegible] the testis a *rich network* [illegible] The angiogram was prepa[illegible] maximum The vasculature [illegible] caput epididymis is hazy × 4

Fig 6 114 hours after the cadmium injection The anastomosis between the testicular and vasal arter[illegible] ow) There is extravasation [illegible] d the vessels of the cauda [illegible]

F g [illegible] trast medium has leaked to [illegible]

Fig 8 [illegible] ustrated Their course and [illegible] in those of the original vessels (compare with Fig 1) The testis is fully involuted at this stage × 4


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6


7


8

the number of visible intratesticular arterial branches was obviously decreased (Fig 2) Filling of the testicular veins with the contrast medium was negligible The vascular bed of the caput epididymis was extensively filled At 24 hours after the treatment a conspicious absence of small intratesticular arteries was observed (Fig 3) The venous return of the contrast medium was almost completely obstructed making visualization of both the testicular and spermatic artery possible The anastomosis between the testicular and vasal arteries was readily visible 2 to 3 days after cadmium injection the internal testicular artery was completely blocked (Figs 4 5 and 6) The venous return was likewise arrested but some anastomotic retrograde venous filling seemed to occur in the hilar area (Fig 4)

The vascular bed of the caput epididymis became more dense and coarse up to 66 hours after the cadmium administration Later on extravasation of the contrast medium was observed (Fig 6) The cauda epididymis was virtually unaffected within the first 66 hours After 24 hours a new network of small vessels made its appearance in the area between the cauda epididymis and the lower pole of the testis A similar network of new vessels was seen in the caput epididymis

After a time lapse of 5 days or more from the cadmium exposure the anastomosis between the internal testicular and vasal arteries was poorly filled with the contrast medium (Fig 5) At the 11th day leakage of contrast medium to the testis parenchyme was always noticed (Fig 7) even at an infusion pressure of 100 mmHg The vascular bed of the cauda epididymis gradually became disorientated the capillary loops forming coarse bundles instead of a fine network (Fig 6)

One month after the cadmium administration a reappearance of new vasculature in the testis was evident (Fig 8) The course and distribution of the new blood vessels did not however correspond to those of the original testicular vessels At this stage the testis was maximally involuted

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Figs 5–8

Fig 5 66 hours after the cadmium injection The testicular parenchyme do not contain a single bl[illegible] l ves[illegible] the testis a r ch networ[illegible] Th[illegible]

Fig 6 [illegible]

Fig [illegible]

Fig 8 [illegible] d to [illegible] and [illegible] 1)



Figs 9 10

Fig 9 Microangiogram of a normal testis section There are capillary loops distributed around the tubules and some coarser vessels in the intertubular spaces × 80

Fig 10 Microangiogram from a testis 6 hours after the cadmium injection Most of the vessels are not filled with the contrast medium × 80

2 *Microangiographic Observations*

In the normal testis the intertubular vessels formed vascular trunks running parallel to one another From these trunks a network of capillaries was seen distributed around the tubules (Fig 9) 6 hours after the cadmium injection both the capillary loops and the main vascular trunks were unevenly or uncompletely filled with the contrast medium



Figs 11 13

Fig 11 The normal appearance of testicular blood vessel after injection of "Thorotrast" to a living animal A few radial veins are visible in addition to the main testicular arteries × 4

Fig 12 3 hours after an injection of cadmium chloride the arteries are almost similarly filled with "Thorotrast than in the normal testis (Fig 11) while the veins do not contain the contrast medium × 4

Fig 13 6 hours after the cadmium injections neither arteries nor veins are filled with thorotrast × 4

(Fig 10) After 14 hours and later only the largest vessels were open and they were often seen to terminate sharply

3 *Experiments with Thorotrast*

The normal appearance of testicular arteries is shown after Thorotrast injection to a living animal in Fig 11 The internal testicular artery with its main branches were well visualized Some veins were seen to run radially towards the hilus 3 hours after the cadmium administration some of the testes were completely lacking the contrast medium while the opposite testis could have almost normal arterial pattern although the veins were never visualized (Fig 12) 6 hours or more after the cadmium exposure no contrast medium was ever seen in the testicular vessels (Fig 13)

DISCUSSION

There are two theories for the explanation of the cadmium induced testicular lesions the degeneration and subsequent necrosis of the seminiferous tubules could be produced through a primary action of

cadmium in the germinal epithelium, or they may follow secondarily after a primary toxic action in the testicular blood vessels (*Parizek* 1960) While the former theory has been supported only by the experiments demonstrating protection of the testis lesions by zinc (*Parizek* 1960, *Gunn et al* 1961), most of the recent investigators have subscribed to the vascular theory (*Gunn et al* 1963, *Chiquoine* 1964, *Mason et al* 1964) The angiographic observations presented in this paper also support this latter theory The site of the vascular injury has not been, however, clearly defined *Gunn et al* (1963) have shown that the main effects of cadmium will be found in the spermatic artery and pampiniform plexus Our observation of a diminished filling of the pampiniform plexus with the contrast medium at 24 hours after the cadmium injection is in agreement with these findings and is presumably an indication of thrombosis and inflammatory changes in the veins

Changes in the filling of the testicular artery and its branches was observed not earlier than 38 hours after cadmium exposure These were obviously due to increased intratesticular pressure since the extratesticular branches of the testicular artery were still well filled as were the intratesticular branches when the injection pressure was increased The high resolution of the microangiographic technique used in the present study made observation of the early effects of cadmium on the capillary bed of the testis possible and our results fully confirm the electron microscope studies of *Chiquoine* (1963, 1964), which revealed early changes in the endothelium in the testis The *in vivo* experiments made using Thorotrast as a X-ray contrast medium have given further support for the theory of a primary vascular injury

The late appearance of a cadmium injured testis has been little studied, because most of the investigators have considered the lesion as irreparable However, *Allanson & Deanesly* (1962) have reported of some repair in the germinal epithelium after 4 months, and most other investigators agree that the interstitial tissue may well restore its vitality and, perhaps, even proliferate The re-establishment of the testis vasculature observed in the present study would apparently take place by virtue of vital intertubular tissue An important source for the new blood vessels was the cauda epididymis, whose vasculature remains unaffected during the first 5 days after the cadmium injection The earlier investigators have not observed any changes in the cauda epididymis, which receives its blood supply through the vasal artery It may be, however, that the cadmium-induced testicular lesion provokes a retraction of the testes which may later induce changes in the vasculature of the caudal part of the epididymis

SUMMARY

With the aid of low tube voltage and high resolution photographic emulsion, the vascularization of the rat testis and epididymis could be

followed to capillary level A single subcutaneous injection of cadmium chloride (0 03 mM/kg) caused closure of the intratesticular capillary network which was observable after 14 hours in ordinary angiographs and after 6 hours when *microangiographic technique* was applied Experiments with Thorotrast injections into living animals showed that the testicular circulation was partly arrested 3 hours after the cadmium administration A re establishment of testicular vasculature was seen beginning 4 weeks after the cadmium exposure The new blood vessels originated partly from the hilar area of the testis, but they took a different course and distribution from the original testis vessels

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"CEREBRAL THROMBOANGIITIS OBLITERANS"

Report of a Case with Discussion of Pathogenesis

By

ANSGAR TORVIK and JENS HOGNESTAD

Received 22 viii 64

During the last years the existence of Buerger's disease as a specific vascular disorder has been seriously questioned by several authors (*Fisher* 1957, *Gore & Burrows* 1958, *Wessler et al* 1960) With the possible exception of the so-called "acute lesion", originally described by *Buerger* (1914–15), the morphological changes in the vessels are not in any way different from thrombo-embolic lesions in general The acute lesion, in which the specific feature should be microabscesses and multinucleated giant cells in the thrombi, has been reported only in few cases (see *Wessler et al* 1960, *McKusick et al* 1962) On the other hand, both multinucleated giant cells and "microabscesses" have been seen during the organization of experimentally produced arterial thrombi in rabbits (*Jorgensen* 1965)

In most cases the diagnosis of Buerger's disease rests upon clinical criteria Progressing signs of ischaemia in the distal parts of the extremities in young men without evidence of atherosclerosis Usually there is a history of excessive cigarette smoking The disease is frequently accompanied by a migrating superficial thrombophlebitis On angiographic examination segmental occlusions are found in medium-sized and small arteries, and roentgenologic criteria of atherosclerotic lesions are lacking

From a pathological point of view it must be agreed that little is left which justifies the classification of this syndrome as a distinct entity On the other hand it may represent an oversimplification to consider all cases merely as thrombo-embolic complications in atherosclerotic subjects This may particularly be the case when arteries and veins are affected simultaneously

The case to be presented showed evidence of progressing thrombosis which probably can not be explained either by atherosclerosis or by a specific disease of the vessel walls The main lesions in the case were located in the central nervous system, and both clinical symptoms and pathological findings correspond to the syndrome known as "cerebral thromboangiitis obliterans"

"Thromboangiitis obliterans" of the brain was first described by *Jager* (1932), *Spatz* (1935) and *Lindenberg & Spatz* (1939). In certain cases of cerebral infarcts these authors noted small white occluded arteries in the meninges overlying the infarcted areas, while the large arteries on the base of the brain were open and showed no atherosclerosis. From the observation of 22 cases *Lindenberg & Spatz* described two broad types of lesions. Type I showed irregular areas of infarction within the territory of one or more of the major cerebral arteries. In type II bilateral symmetrical "granular atrophy" of the cortex was found in sickleshaped zones along the entire convexity of the brain. These changes were confined to the border zones between the anterior and middle and posterior cerebral arteries. On microscopical examination multiple tiny cortical infarcts were found within the areas of granular atrophy. In later reports transitions between the two types have been described (see *Eicke* 1957). Varying degrees of intimal oedema and inflammatory reaction has been found in the vessel walls, and the occlusions *in the meningeal vessels have been variously ascribed to* intimal proliferation alone, or to thrombosis with organization (*Eicke* 1957).

In many reported cases of this disease the carotid arteries were occluded by thrombi. Furthermore it should be noted that the disease was not always accompanied by symptoms from the extremities.

In an extensive and critical review of the literature on "cerebral thromboangiitis obliterans" *Fisher* (1957) drew attention to the significance of the carotid occlusions which are so frequently reported to accompany the cerebral form of this disease. He presented 5 cases of type I of *Lindenberg & Spatz*, all of which had occlusions of the carotid or middle cerebral arteries. In his opinion the occlusions of the small meningeal arteries resulted from stagnation of the blood flow consequent to the proximal occlusions. He suggested that both the cerebral and peripheral types of Buerger's disease were secondary to atherosclerotic occlusions of the major proximal arteries.

The following case showed the characteristic lesions of "cerebral thromboangiitis obliterans" without occlusion of the major cerebral arteries. It is presented to introduce a possible aetiology other than atherosclerosis in cases of progressing thrombosis in young persons.

CASE REPORT

History. In 1952, at the age of 43, a male tobacco factory worker (and cigarette smoker) was admitted to the Surg[illegible] chron[illegible]

[illegible]

From 1959 he began to show mental changes. He became jealous and suspicious and gradually exhibited signs of mental deterioration. From January 1962 the

right arm and hand gradually became weak clumsy and numb Later the same symptoms appeared also in the left hand Small twitchings of the muscles in the arms were noted He was admitted to the Neurological Department Ullevål Hospital in October, 1962 On admission his blood pressure was 190/125 The pulse was normal No signs of cardiac insufficiency were found at admission or later in the course of the disease There were no petechiae of the skin or mucous membranes He had a poor memory decreased capacity of concentration and a marked right left confusion The vision was reduced to finger counting at 20 cm's distance The pupillary reactions were preserved Ophthalmoscopy showed moderate hypertensive vascular changes in the retina There was a severe paresis of the fingers and shoulder on the right side and a moderate paresis on the left side The supraspinatus muscles were atrophic bilaterally Occasional fasciculations of the muscles of the right arm were noted All tendon reflexes were moderately increased The right plantar reflex was normal There was a definite bilateral finger agnosia which was most pronounced on the right side Cutaneous and deep sensation was stated to be normal On electromyographic examination of the muscles of the right upper extremity numerous fasciculations were observed During the following weeks he became increasingly demented with confusion restlessness and hallucinations His vision gradually got worse Further detailed neurological examinations are lacking During the last days he was comatose He died from bronchopneumonia on December 3 1962 During the entire course of the disease there was a gradual progression of the symptoms without acute episodes

The following laboratory examinations were performed in October 1962 Haemoglobin 12 5 g % Erythrocytes 4 25 mill./mm³ Leucocytes 5500/mm³ Prothrombin 95 % Sedimentation rate 40 mm/hr Creatinine 1 4 mg % Serum electrophoresis showed normal values Liver function tests and differential counts of blood smears were normal Blood platelets were not counted and no coagulation studies were performed The spinal fluid was normal

Post mortem examination —The left leg was amputated below the knee and the right 1st toe showed a chronic ulceration The aorta and the large arteries showed moderate atherosclerosis The left femoral artery was totally occluded by an old organized thrombus No occlusions were found in the arteries of the right leg The deep veins of the legs were patent The heart weighed 580 g There was an old organized thrombus in the right coronary artery and an old myocardial infarct in the interventricular septum No endocardial thrombi were found

Both carotid arteries were taken out in their full length The left carotid artery showed only small patches of atherosclerosis At the bifurcation on the neck the lumen of the right internal carotid artery was narrowed by an atherosclerotic plaque to about one half of its original diameter Superimposed on this plaque was a fresh mural thrombus of 1–2 mm thickness, which on microscopic examination appeared to be only a few days old The leucocytes in the thrombus showed no signs of degeneration The cervical part of the vertebral arteries was not examined The arteries on the base of the brain and the intracranial parts of the vertebral arteries were completely free of atherosclerosis The configuration of the circle of Willis was normal

On the convexity of the brain two symmetrical zones of old and recent infarction were seen, extending from the frontal to the occipital poles Numerous small, occluded white arteries were seen in the meninges overlying the areas of infarction (Fig 1) The infarcts were mainly confined to the cortex and white matter along the border zones between

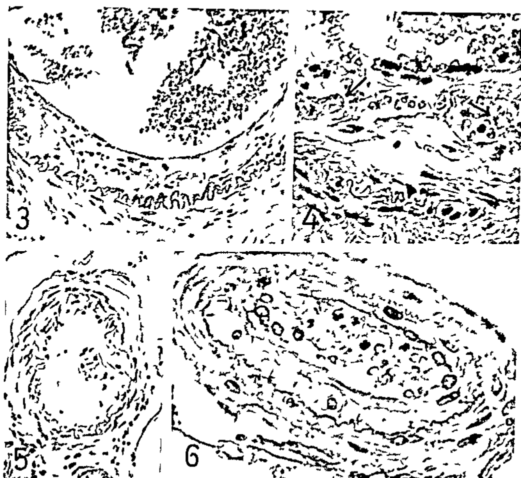


Fig 1
Small white occluded vessels in the meninges over cortical infarcts

the anterior and middle cerebral arteries (Fig 2) Smaller infarcts were seen along the lateral sides of the temporal lobes along the border zones between the middle and posterior cerebral arteries Scattered small cortical infarcts were also seen on the medial surface of the brain along the median longitudinal fissure and on the medial surface



Fig 2
Schematic drawings of the cerebral hemispheres showing the distribution of the infarcts (stippled)



Figs 3-6

Fig 3 Subintimal fibrosis of small meningeal artery Haematoxylin and eosin × 210

Fig 4 Subintimal fibrosis of small meningeal artery due to organizing mural thrombus Small remnants of the thrombus are seen at arrows Haematoxylin and eosin × 510

Fig 5 Subtotal fibrous occlusion of small meningeal artery Haematoxylin and eosin × 210

Fig 6 Subintimal collections of lipid macrophages in small meningeal artery Haematoxylin and eosin × 510

of the occipital lobes The calcarine cortex was almost completely destroyed Most of the infarcts were old but areas of recent and old infarction were often diffusely intermingled

The spinal cord and nerve roots were grossly normal Apart from a bronchopneumonia no significant gross findings were made in other organs

Microscopic examination of the brain confirmed the presence of infarcts of varying age, from a few days to months or years Old and recent infarcts were diffusely intermingled In addition to the lesions which were seen on gross examination, numerous scattered tiny infarcts were found in other areas of the cerebral cortex and in the cere-



Figs 7 10

Fig 7 Organizing thrombus in meningeal artery (right) Platelet thrombus in smaller artery (left) Haematoxylin and eosin × 210

Fig 8 Fibrin thrombus in small intracerebral vein Lendrum s stain × 80

Fig 9 Fibrous occlusion of meningeal artery Small recanalized vessels are occluded with fibrin PTAH × 210

Fig 10 Recent platelet aggregates in spinal meningeal arteries Haematoxylin and eosin × 210

bellum and brain stem The spinal cord showed no infarcts Two intra spinal nerve roots in the lumbar region were fibrotic and shrunken without remnants of nerve fibers

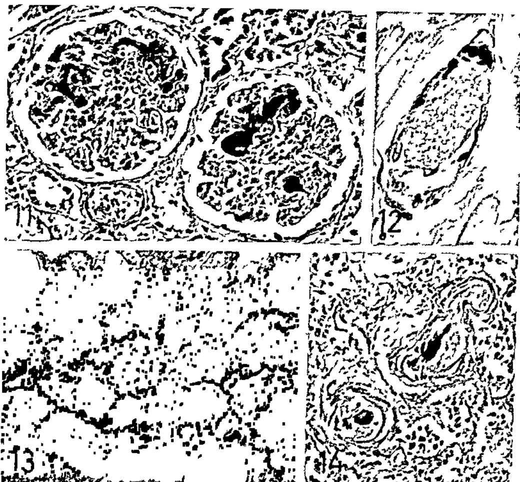
In the meninges, and also in other organs, concentric fibrous intimal thickening of the small arteries was frequently observed (Fig 3) These changes were often seen in arteries with outer diameters down to 100 μ Lipid macrophages were occasionally seen within the thickened intima (Fig 6) The endothelial cells sometimes appeared swollen

pr[illegible]

an[illegible]

co[illegible] organized thrombi (Figs 7–10) Judged by the PTAH and Lendrum stains most of the recent thrombi were composed of platelets with little or no fibrin but occlusions rich in fibrin were also seen (Fig 8)

In vessels with recent thrombi the walls were often infiltrated with



Figs 11–14

Fig 11 Fibrin precipitates in the glomeruli of the kidney. PTAH × 210
Fig 12 Platelet aggregate in small myocardial vein Haematoxylin and eosin × 510
Fig 13 Fibrin precipitates in pulmonary capillaries PTAH × 80
Fig 14 Mixed aggregates of platelets and fibrin in vessels in the spleen PTAH × 210

polymorphonuclear leucocytes but there was no evidence of a primary inflammatory process in the vessels Most of the occlusions were found in perfectly healthy vessels

In the brain the infarcted areas and the overlying meninges were studded with these small occluded vessels Many recent and old occlusions were also found diffusely scattered in the meninges over the entire brain and spinal cord without infarcts in the underlying tissue (Figs 7–10). Fresh thrombi without infarcts were also seen in small vessels within the brain and spinal cord Presumably these were too recent to give morphologic changes in the surrounding tissue

Further, all other examined organs contained innumerable thrombi in small arteries, capillaries and veins (Figs 11–14) The occluded vessels were rarely more than 500 μ in diameter Most occlusions were recent and consisted of platelet aggregates and fibrin Others were older

and showed evidence of organization Such occluded vessels were found in the kidneys, spleen pancreas, liver, heart, lungs, striated muscle and the retina of the eye

In the heart several small microinfarcts of varying age were found in addition to the old infarcts which were seen on gross examination Sections from the right soleus muscle and from *the right supraspinatus* were normal except for several occluded small vessels Striated muscle around the posterior tibial artery on the lower part of the right calf showed a definite neurogenic atrophy

The left femoral artery was markedly atherosclerotic and the lumen was filled with a completely organized thrombus Sections from the radial artery, the right posterior tibial and the right dorsalis pedis arteries were normal

DISCUSSION

The present case was unusual in several ways Clinically it was remarkable by a gradual onset and progression of neurologic symptoms In addition there were signs of progressing ischaemia of both legs While the symptoms from the left leg can be explained by a thrombotic occlusion of the left femoral artery, no occlusions of the major arteries of the right leg were found

The basic pathologic lesions in the nervous system were infarcts of varying age which mainly were located to the "water shed areas" of the brain, *i e* to the border zones between the areas of supply of the major intracerebral arteries (*Fisher* 1954, *Zulch* 1961) Numerous small occluded white vessels were found in the meninges over the infarcted areas These findings are typical of the so-called "thromboangiitis obliterans of the brain Due to the localization of the infarcts the case might [illegible]

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[illegible] infarcts with this distribution are frequently found after occlusions of the carotid arteries (*Fisher* 1954, *Torvik & Jorgensen* 1964) [illegible]

[illegible] appearance this was not more than a few days old Unfortunately the cervical portion of the vertebral arteries was not examined but the cerebral lesions can hardly be explained by vertebral occlusions alone It should be noted that there was no clinical evidence of cardiac failure or hypotensive episodes, and that the clinical course of the disease was steadily progressive

The most striking findings on microscopical examination were innumerable occluded small vessels which were found in all organs in various stages of organization Undoubtedly they played a rôle for the precipitation of the infarcts in the brain and also in the myocardium

Presumably the peripheral pareses with fasciculations can be explained by ischaemic lesions of the intraspinal nerve roots and peripheral nerves

Although thrombo embolic occlusions of small vessels are occasionally seen in cerebral infarcts after occlusions of major cerebral arteries, their unusual number and general distribution in this case suggest that they were of special pathogenetic significance Since they were equally frequent in arteries and veins, it is likely that the majority of them were thrombotic and not embolic

No evidence of primary inflammatory lesions of the vessels was found As described above, the small arteries often showed fibrous intimal thickening Occasionally subendothelial collections of lipid macrophages were also found (Fig 6) Such changes are indistinguishable from atherosclerotic lesions which sometimes are found in small meningeal arteries, particularly in cases of hypertension and diabetes However, the heaviest changes in such cases are invariably found in the major cerebral arteries All arteries on the base of the brain of this case were free of atherosclerosis while the intimal thickening was found in vessels with diameters down to 100 μ Furthermore, remnants of thrombotic material was occasionally seen within the intimal thickening (Fig 4), and all transitions were found between intimal thickenings and complete fibrous occlusions of the lumen (Figs 3–5) It seems most likely, therefore, that these changes were caused by organization of mural thrombi Similar vascular changes with fibrous intimal thickening and subintimal collections of lipid macrophages have been seen during organization of experimentally produced thrombi in rabbits (*Hand & Chandler* 1962, *Jorgensen* 1964)

Most of the recent vascular occlusions, both in the nervous system and in other organs, were found in perfectly healthy vessels In view of this, and of their generalized distribution and unusual number, it is likely that they were caused by a disorder of the clotting mechanisms, rather than by a disease of the vessel walls Unfortunately, no relevant laboratory studies were made which could elucidate these mechanisms Since most occlusions were found in the smallest vessels where the circulation is relatively slow, this may play a rôle for the precipitation of the thrombi Similarly, the localization of the infarcts along the "water-shed areas" in the central nervous system points to the importance of haemodynamic factors in the precipitation of the lesions (*Zulch* 1961)

As mentioned in the introduction, there is little evidence that Buerger's disease represents a specific disorder of the vessel walls It may be doubtful, therefore, whether the retention of the original name attached to this clinical syndrome serves any purpose Undoubtedly, many of the reported cases are examples of thrombo-embolic phenomena in young atherosclerotic subjects In others such as the present case, there may be a progressing thrombosis from other causes

Several authors have suggested that there may be an abnormality of the coagulation mechanisms in these patients (*Aygaard & Brown* 1937, *Theis & Freeland* 1939, *Takats* 1943, *Hagedorn & Barker* 1948, *Gore & Burrows* 1958, *Ende* 1963) However, both the biochemical and pathologic findings in support of this hypothesis have been vague In the present case the pathological findings speak rather firmly in favour of a clotting abnormality as the underlying disorder The case is presented in the hope that attention may be focused on the platelet function and clotting mechanisms in future cases of obscure progressing thrombosis

SUMMARY

A case of so called cerebral thromboangiitis obliterans in a 53 year-old man is reported He developed signs of ischaemia of the legs from the age of 43 During the last 3 years he became increasingly demented He gradually developed bilateral pareses of the upper extremities, fasciculations of the arms, right left confusion, finger agnosia, and blindness

At autopsy bilateral symmetric infarcts of varying age were found in the brain along the border zones between the major cerebral arteries Overlying these areas were numerous small, white, occluded meningeal vessels Old vascular lesions were found in two intraspinal nerve roots No occlusions were found of the major cerebral arteries The left femoral artery was occluded by an organized thrombus On microscopic examination innumerable occlusions of varying age were found in small arteries capillaries and veins in all examined organs The occluded vessels rarely exceeded 500 μ in diameter The recent occlusions were composed of platelet aggregates and fibrin No evidence was found of a primary disease of the walls of the occluded vessels

The progressing thrombosis in this case was probably caused by a clotting abnormality of the blood It is suggested that this may be the basic disorder in some cases of so called Buerger's disease

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PREMITOTIC UPTAKE OF TRITIATED THYMIDINE BY MAST CELLS

By

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Received 8 x 64

Mast cells play an important role in the physiology of connective tissues Their capability of synthesis and release of ground substance components which may bind tissue water places these cells in a key position in the mechanism of growth, regeneration, and repair as well as in several pathological processes

It has been a matter of dispute how the tissue mast cells regenerate, whether they divide in a non granular phase of early development or actually as granular mast cells In adult organisms, mitoses have very rarely been observed in cells fulfilling the histological criteria of mast cells (*Downey* 1913, *Padawer* 1960) This may be due to the granular cytoplasmic material partially or entirely covering the nucleus After degranulation of mast cells by component 48 80, *Hunt & Hunt* (1957) found a reasonable number of mitoses in mast cells

W[illegible]

acti[illegible]

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phe[illegible] of mitotic division can be observed It maintains the label in the course of mitosis and passes it on to the next generation of cells

A correct interpretation of the experimental results obtained by means of autoradiography requires i e that the radiation effect due to incorporation of a labelled substance is low, and the autoradiographic resolution high The use of moderate doses of tritiated thymidine as a DNA precursor seems to meet these requirements DNA is almost electively labelled and the low beta ray energy of tritium ensures high resolution of the autoradiograms However, self absorption of the soft

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beta radiation emitted by tritium in the tissue proper makes the interpretation of these autoradiograms very difficult

EXPERIMENTAL

White female mice of the strain St/EH six weeks old were painted four times with 0 5 ml of a 0 5 per cent benzene solution of the carcinogenic hydrocarbon 9 10 dimethyl 1 2 benzanthracene After six to seven weeks 10 per cent of the painted mice had developed one or more papillomas on their back skin In histological sections of the induced skin tumours dense accumulation of mast cells was demonstrated

The tumour bearing mice were injected intraperitoneally with 5 microcuries of tritiated thymidine per gram body weight The injections were given 8 to 10 days after at least one papilloma had been observed on the back skin

One half hour and up to 7 days after the injection the animals were sacrificed the tumours together with surrounding skin were excised and fixed in a solution consisting of absolute alcohol 85 per cent formaldehyde 10 per cent and glacial acetic acid 5 per cent Thereafter the tissue was embedded in paraffin and sections were cut at seven micra s thickness After deparaffinization autoradiograms were made using Kodak AR 10 stripping film Double exposures were made in all cases lasting 14 days and 28 days respectively After photographic processing of the autoradiograms the tissue was stained with toluidine blue (0 5 per cent in 40 per cent ethyl alcohol) and mounted The autoradiograms were evaluated by visual grain counting using an oil immersion objective (100 ×) and an eye piece (10 ×) into which a net micrometer with 10 × 10 small squares was inserted The rectangular field was 60 × 60 = 3600 square micra

Three series of experiments were carried out In the first the animals were sacrificed at various time intervals between 0 5 and 20 h after injection of tritiated thymidine In the second series these time intervals were extended from 12 h to 120 h after injection and in the third the animals were sacrificed 1 3 4 6 and 7 days after administration of the labelled precursor

From each animal the 14 days exposure autoradiogram was analysed and at least 1000 mast cells were carefully examined The number of grains over each cell was counted and *whenever practicable it was noted down how many grains were located* over the nucleus the cytoplasm or the boundary region respectively In the following these cells are called labelled regardless of whether the grains observed over the cells are due partly or exclusively to background The term significantly labelled is defined on p 543 The random grain background of each plate was determined by counting the grains in at least 10 fields of 3600 μ^2 adjoining the tissue section or in empty spaces within the tissue In addition grain counts were performed over mast cells in tissue from animals treated like those in the experimental groups [illegible] midine [illegible] as viewed in the microscope—was obtained [illegible] ndomly selected cells with the superimposed small squares of the eye piece [illegible] ometer Since the values thus obtained did not agree t o well with those based on grain counts on blanks (cf p 539) an estimate of the average cell size was also obtained by projecting the cells through the microscope on to a sheet of paper drawing their outlines and weighing the paper clippings Also this method is coarse and subject to systematic errors

QUALITATIVE RESULTS

As discussed in a previous publication (4) the results obtained in the *first series* of experiments seemed to indicate that the degree of labelling of mast cells after injection of tritiated thymidine was almost constant over a period of 20 h post injection On an average 2 per cent of the cells were labelled significantly (cf p 12) It was not possible to distinguish between cytoplasmic and nuclear label A change in the percent

age of labelled cells as a function of time could not be established with certainty

In the *second series*, in which the experimental period was extended over 120 h after injection of the precursor, the percentage of labelled cells increased with time over a period of about 100 h, and heavily labelled cells were observed 5 days after injection of the precursor

The results of the *third series* in which the animals were sacrificed between 1 and 7 days post injection were somewhat different However, even 7 days after injection, heavily labelled mast cells were found in considerable numbers

Limitations of the Method

As mentioned previously (4, 6), the imperfections of the technique used in this study seriously limit the information that can be obtained from the autoradiograms

When tritium is used as a tracer, a 7 μ section represents an infinitely thick layer from which only a small fraction of the beta particles emitted can emerge and reach the emulsion This fraction may be of the order of 1 per cent or less of the disintegrations actually taking place in the sample Therefore, the autoradiograms obtained depict the distribution of the label in the top one, or at best two micra of the section, only

Also from the histological point of view, a tissue section has a number of imperfections First of all, sections are never quite uniform Moreover, the observer is tempted to picture a metachromatically stained mast cell with its clearly outlined nucleus as a thin disc Actually, it is shaped, rather, like a mutilated balloon and the "nucleus" may be located at some depth and covered by a sheet of cytoplasm of varying thickness Consequently, a labelled nucleus 1 or 2 micra removed from the emulsion may appear inactive On the other hand, a thin layer of stained, inactive cytoplasm hiding a "hot" nucleus may be interpreted as 'labelled cytoplasm" A clear-cut attribution of blackening of the emulsion to the underlying structure is possible only provided that both the nucleus and the cytoplasm touch the cutting surface of the section, *i e* that the cell happens to be cut through the nucleus (cf also p 12)

The situation may be improved somewhat by reducing the thickness of the specimen to or below the maximum range of the particles emitted, i e to about 2 μ In this case, the resulting autoradiogram provides a better approximation of the actual distribution of the tracer in the tissue Such an approach was attempted However, when skin papilloma preparations were cut at 2 μ, mast cells were badly torn and granules were smeared out by the microtome knife From the histological point of view, these thin sections were very poor Thus, what may be gained from a more representative autoradiogram will be lost through the

sacrifice of histological detail Therefore, 7 μ sections were used throughout

Qualitative Evaluation of Data

The evaluation of autoradiograms is based on a determination of the number of cells which have given rise to grains in the emulsion, and the number of grains per cell With a 7 μ section, a considerable fraction of the cells viewed in the microscope does not produce any grains at all due to self-absorption of the tritium betas, and therefore, the number of "labelled" cells will necessarily turn out to be too low Correspondingly, the "number of grains per cell"-values are minimum values However, the contribution of random background grains to the total is independent of sample thickness

TABLE 1

mber of Mast Cells with (N) Grains at Different Times after Injection of Tritiated Thymidi
(Series I)

Number of grains	Number of cells total and in per cent											
	0 5 h		2 h		12 h		16 h		20 h		Total	
N	no	%	no	%	no	%	no	%	no	%	no	%
0	716	71 6	878	78 6	607	60 2	299	57 5	710	67 6	3210	68 4
1	146	14 6	136	12 2	208	20 6	96	18 5	189	18 0	775	16 5
2	73	7 3	49	4 4	96	9 5	47	9 0	81	7 7	346	7 4
3	36	3 6	19	1 7	40	4 0	35	6 7	31	3 0	161	3 4
4	10	1 0	11	1 0	24	2 4	10	1 9	17	1 6	72	1 5
5	6	0 6	4	0 4	10	1 0	11	2 1	5	0 5	36	0 8
6–9	9	0 9	4	0 4	11	1 1	16	3 1	10	1 0	50	1 1
0–14	3	0 3	7	0 6	5	0 5	4	0 8	5	0 5	24	0 5
5–19			2	0 2	2	0 2					4	0 1
0–24			3	0 3	4	0 4	1	0 2			8	0 2
5–29			2	0 2			1	0 2	2	0 2	5	0 1
30–34			2	0 2							2	0 04
35–39	1	0 1			1	0 1					2	0 04
	1000		1117		1008		520		1050		4695	
ckground r 100 μ^2	0 87		1 40		0 65		0 39		1 35			
tal	623		503		469		271		485			

Tables 1, 2, and 3 present the counting results of all specimens investigated The number of grains per cell is given at various times after injection of tritiated thymidine The last horizontal rows in each table show the mean random background of each plate, expressed as number of grains per 100 μ^2, and the total number of grains observed when examining, as a rule, 10 fields of 3600 μ^2 each

It appears from Table 1 that there are no striking differences between the distributions of "grains per cell" for the five time intervals

TABLE 2

Number of Mast Cells with (N) Grains at Different Times after Injection of Tritiated Thymidine
(Series II)

Number of grains N	12 h no	12 h %	24 h no	24 h %	48 h a no	48 h a %	48 h b no	48 h b %	72 h a no	72 h a %	72 h b no	72 h b %	96 h a no	96 h a %	96 h b no	96 h b %	120 h no	120 h %
	Number of cells total and per cent																	
0	640	62 7	203	40 4	132	26 3	120	24 0	210	41 3	96	19 2	115	23 0	140	28 0	324	31 9
1	243	23 8	159	31 6	166	33 0	152	30 4	143	28 1	107	21 4	128	25 6	127	25 4	271	26 7
2	70	6 9	57	11 3	88	17 5	82	16 4	60	11 8	94	18 8	85	17 0	96	19 2	147	14 5
3	37	3 6	36	7 2	59	11 8	61	12 2	51	10 0	101	20 2	57	11 4	48	9 6	93	9 2
4	11	1 1	15	3 0	35	7 0	33	6 6	12	2 4	29	5 8	53	10 6	25	5 0	64	6 3
5	3	0 3	4	0 8	11	2 2	24	4 8	5	1 0	37	7 4	21	4 2	20	4 0	37	3 6
6 9	9	0 9	10	2 0	7	1 4	20	4 0	12	2 4	31	6 2	24	4 8	22	4 4	47	4 6
10 14	1	0 1	8	1 6			5	1 0	6	1 2			4	0 8	4	0 8	14	1 4
15 19	4	0 4	4	0 8			1	0 2	1	0 2			7	1 4	8	1 6	9	0 9
20-24	1	0 1	4	0 8	2	0 4			1	0 2	1	0 2	3	0 6	3	0 6	3	0 3
25 29	1	0 1	2	0 4			1	0 2					2	0 4	1	0 2	1	0 1
30 34					1	0 2			3	0 6	1	0 2			1	0 2	1	0 1
35 39															2	0 4	1	0 1
40			1	0 2	1	0 2	1	0 2	5	1 0	3	0 6	1	0 2	3	0 6	3	0 3
	1020		503		502		500		509		500		500		500		1015	
Background per 100 μ^2	0 82		1 08		2 24		2 02		1 10		3 02		3 10		1 19		1 43	
Total	324		387		808		729		395		1088		1116		428		1029	

sacrifice of histological detail. Therefore, 7 μ sections were used throughout.

Qualitative Evaluation of Data

The evaluation of autoradiograms is based on a determination of the number of cells which have given rise to grains in the emulsion, and the number of grains per cell. With a 7 μ section, a considerable fraction of the cells viewed in the microscope does not produce any grains at all due to self-absorption of the tritium betas, and therefore, the number of "labelled" cells will necessarily turn out to be too low. Correspondingly, the "number of grains per cell"-values are minimum values. However, the contribution of random background grains to the total is independent of sample thickness.

TABLE 1

Number of Mast Cells with (N) Grains at Different Times after Injection of Tritiated Thymi[dine] *(Series I)*

Number of grains	Number of cells total and in per cent											
	0.5 h		2 h		12 h		16 h		20 h		Total	
N	no	%	no	%	no	%	no	%	no	%	no	%
0	716	71.6	878	78.6	607	60.2	299	57.5	710	67.6	3210	68.4
1	146	14.6	136	12.2	208	20.6	96	18.5	189	18.0	775	16.5
2	73	7.3	49	4.4	96	9.5	47	9.0	81	7.7	346	7.4
3	36	3.6	19	1.7	40	4.0	35	6.7	31	3.0	161	3.4
4	10	1.0	11	1.0	24	2.4	10	1.9	17	1.6	72	1.5
5	6	0.6	4	0.4	10	1.0	11	2.1	5	0.5	36	0.8
6–9	9	0.9	4	0.4	11	1.1	16	3.1	10	1.0	50	1.1
10–14	3	0.3	7	0.6	5	0.5	4	0.8	5	0.5	24	0.5
15–19			2	0.2	2	0.2					4	0.1
20–24			3	0.3	4	0.4	1	0.2			8	0.2
25–29			2	0.2			1	0.2	2	0.2	5	0.1
30–34			2	0.2							2	0.0
35–39	1	0.1			1	0.1					2	0.0
	1000		1117		1008		520		1050		4695	
Background per 100 μ^2	0.87		1.40		0.65		0.39		1.35			
Total	623		503		469		271		485			

Tables 1, 2, and 3 present the counting results of all specimens investigated. The number of grains per cell is given at various times after injection of tritiated thymidine. The last horizontal rows in each table show the mean random background of each plate, expressed as number of grains per 100 μ^2, and the total number of grains observed when examining, as a rule, 10 fields of 3600 μ^2 each.

It appears from Table 1 that there are no striking differences between the distributions of "grains per cell" for the five time intervals

was made to obtain additional information on this point from a statistical analysis of the data

First, the percentage of significantly labelled cells for each time interval between injection and sacrifice was calculated. Second, a comparison was made of the degree of labelling of the cytoplasm with that of the cell nuclei. Since the visual inspection of the autoradiograms gave the impression of a preponderance of blackened grains over the cytoplasm, although cells with a heavily labelled nucleus were observed, it seemed worth while to study the localization of grains over different cell regions, *viz.* cytoplasm, nucleus, and a boundary zone, both as a function of time and of the total blackening of the cells

STATISTICAL EVALUATION

Frequency of Unlabelled and Significantly Labelled Cells

The calculation of the percentage of significantly labelled cells from autoradiographic data requires *ad 1)* the determination of the number of cells with and without blackening, and *ad 2)* the calculation of the frequency of a given "blackening", *i.e.* "number of grains per cell" due to the random background in the emulsion. While variations of the background (f. ex. expressed as grains per 100 μ^2) are assumed to follow a Poisson distribution, variations in cell size cause the distribution of "grains per cell" to deviate from Poisson. The variance of the resulting distribution becomes equal to the variance of the Poisson distribution plus a contribution due to the variance of the cell area.

Variations of the background were found to be somewhat larger than corresponding to a Poisson distribution. However, the variance was only increased by 0.0015 which is negligible as compared to the contribution due to the variation in cell area.

The mean cell area was estimated visually to be 47 μ^2. Using a blank specimen, *i.e.* inactive tissue covered by emulsion, exposed, processed, and evaluated in the same manner as discussed previously, the background of the blank plate was determined to be 0.436 grains per 36 μ^2 and the mean grain count per cell was 0.848. This leads to a mean cell area of 70 μ^2. The standard deviation of the cell area measurements was 45 per cent on an average. The total variance is thus

$$\sigma^2 = \nu + \frac{1}{\lambda} \nu^2 \qquad (1)$$

where ν is the mean number of grains per cell and $1/\lambda$ is the ratio between the variance of the cell area and the square of its mean value. This distribution can be described by a so-called negative binomial function (cf. *Feller* 7). With a standard deviation in the cell area measurements of 45 per cent, we arrive at $1/\lambda = 0.20$ or $\lambda = 5$, which has been used

listed The scatter about the mean values is as large as would be expected Also the frequency of cells with zero grains is fairly constant, and variations in random background have no marked effect on the observed grain distribution over cells

Table 2 contains the corresponding data for the second group of animals In the preparations representing 48 h, 72 h and 96 h post injection, a total of 1000 mast cells were examined, but observations were made on 500 cells from each of two animals injected and sacrificed at the same time A comparison of Table 1 with Table 2 reveals that the percentage of labelled cells increases with time between 24 h and 120 h post injection, and cells producing more than 15 grains are seen more often 96 h after injection than at any other time

TABLE 3

Number of Mast Cells with (N) Grains at Different Times after Injection of Tritiated Thymidine (Series III)

Number of grains	Number of cells total and in per cent									
	24 h		72 h		96 h		144 h		168 h	
N	no	%	no	%	no	%	no	%	no	%
0–4	980	94 8	1197	97 3	1068	92 9	1280	98 5	1394	96 9
5	18	1 7	13	1 1	31	2 7	9	0 7	16	1 1
6–9	24	2 3	14	1 1	28	2 4	6	0 5	17	1 2
10–14	9	0 9	3	0 2	9	0 8	4	0 3	7	0 5
15–19	3	0 3	3	0 2	6	0 5			1	0 1
20–24					1	0 1	1	0 1	3	0 2
25–29					1	0 1				
30–34					3	0 3				
35–39										
40–					2	0 2				
	1034		1230		1149		1300		1438	
Background per 100 μ^2	0 43		0 40		0 43		0 44		0 67	
Total	154		143		154		158		241	

In the study of the last group sacrificed up to 7 days post injection, emphasis was placed on the observation of significantly labelled cells

It appears from Table 3 that this series shows a smaller percentage of labelled cells at all times and no obvious trend as a function of time However, it does confirm the observation made on group II that heavily labelled cells are to be found 7 days post injection, they seem to be most numerous about 96 h after administration of the precursor

The results described above confirm that—after injection of a labelled DNA precursor—labelled mast cells are to be found in the tissue It was expected that blackening of the emulsion would be located predominantly over cell nuclei Since imperfections of the method make the topographic interpretation of grain patterns ambiguous, an attempt

TABLE 6

Frequencies of Zero Grains per Cell and of Labelled Cells as a Function of Background

1	2	3	4	5	6	7	8	9		10
	Number of background grains per			Frequency of zero grains				% labelled cells		
Time (h)	100 µ² (obs)	47 µ³ cell (calc)	70 µ³ cell (calc)	Obs %	Calc from 3 %	Calc from 4 %	Signif limit	no	%	Cells with > 10 grains %
0 5	0 87	0 408	0 608	71 6	68	57	6–	13	1 3	0 4
2	1 40	0 657	0 980	78 6	54	41	7	17	1 5	1 4
12	0 65	0 307	0 458	60 2	74	65	5	33	3 3	1 0
16	0 38	0 178	0 265	57 5	84	77	4–	43	8 3	1 2
20	1 35	0 634	0 945	67 6	55	42	7–	14	1 3	0 7
12	0 82	0 386	0 574	62 7	69	58	6–	16	1 6	0 7
24	1 07	0 505	0 754	40 4	62	49	6–	29	5 8	3 8
48	2 24	1 056	1 575	26 3	38	25	9	6	1 2	0 8
48	2 02	0 953	1 421	24 0	42	29	9	10	2 0	1 6
72	1 10	0 516	0 770	41 3	61	49	6–	28	5 5	3 1
72	3 02	1 422	2 120	19 2	29	17	11–	5	1 0	1 0
96	3 10	1 458	2 175	23 0	28	16	11–	15	3 0	3 4
96	1 19	0 559	0 834	28 0	59	46	7–	33	6 6	4 4
120	1 43	0 673	1 004	31 9	53	40	7–	60	5 9	3 2
24	0 4	0 188	0 281		83	76	4–	54	5 2*	1 2
72	0 4	0 187	0 279		83	76	4–	33	2 7*	0 5
96	0 4	0 201	0 300		82	75	4–	81	7 1*	1 9
144	0 43	0 207	0 308		82	74	4–	20	1 5*	0 4
168	0 67	0 315	0 470		74	64	5–	44	3 1	0 8

in the following calculations In view of the inaccuracy of the cell area determinations, these calculations must be taken with some reservation It follows from the negative binomial function that

$$\beta_N = \left(1 + \frac{\gamma}{\lambda}\right)^{-\lambda} (-1)^N \binom{-\lambda}{N} \left(1 + \frac{\lambda}{\gamma}\right)^{-N} \qquad (2)$$

The distribution of the number of grains per cell for the blank plate was calculated from equation (2) using $\lambda = 5$ and the observed average of 0 848 grains per cell It appears from Table 4 that the observed frequencies of occurrence of N grains per cell are in very good agreement with the calculated frequencies Differences apparent in the table are not significant

TABLE 4

Observed and Calculated Frequency of (N) Grains per Cell on a Blank Plate

(N)	Obs	Calc	Obs calc	(O C)²/C
0	234	239	—5	0 10
1	169	155	14	1 27
2	58	68	—10	1 47
3	28	26	2	0 15
4	5	9	—4	1 78
5	6	3	3	3 00
≥ 6	1	1	0	0 00
	501	501	0	7 77 f = 5 not significant

Mean 0 848

TABLE 5

Frequency of (N) Grains per Cell as a Function of Background

(N)	Background in grains cell area			
	0 5	1 0	1 5	2 0
0	62 09	40 19	26 92	18 59
1	28 22	33 49	31 07	26 57
2	7 70	16 75	21 52	22 77
3	1 63	6 51	11 59	15 18
4	0 30	2 17	5 35	8 67
5	0 05	0 65	2 22	4 46
6	0 01 { 0 01	0 24 { 0 18	1 31 { 0 85	3 65 { 2 12
7	—	0 05	0 31	0 95
8	—	0 01	0 11	0 41
9	—	—	0 04	0 17
10–14	—	—	0 02	0 11
	100 00	100 00	100 00	100 00

up to 9 grains per cell, and therefore 'significantly labelled cells" on this plate are those with 10 grains or more On the autoradiogram with the lower background, the observed frequency of 5 grains per cell is 10 times higher than that due to background, and 99 9 per cent of the cells with ≥ 6 grains are significantly labelled In the evaluation of the present data, a cell was considered 'significantly labelled" if the blackening observed immediately above the cell had less than 0 1 per cent chance of being produced by background radiation This blackening i e this 'number of grains' is termed "significance limit and is listed for each autoradiogram studied in column 8 of Table 6

TABLE 8

Localization of Silver Grains over Nucleus Cytoplasm and Boundary of Mast Cells

(1)	NCB (2)	Series I Obs (3)	Series I % (4)	Series II Obs (5)	Series II % (6)
S=1	N 100	192	24 8	188	12 6
	C 001	469	60 5	1141	76.3
	B 010	114	14 7	167	11.2
		775	100 0	1496	100 1
S=2	002	179	51 7	509	65 3
	011	30	8 7	79	10 1
	020	18	5 2	38	4 9
	101	68	19 7	101	13 0
	110	23	6 6	21	2 7
	200	28	8 1	31	4 0
	Number of cells	346	100 0	779	99 9
	N	147	21 2	184	11 8
	C	456	65 9	1198	76 9
	B	89	12 9	176	11 3
	Number of grains	692	100 0	1558	100 0
S=3	003	89	55 3	298	54 9
	012	6	3 7	55	10 1
	102	22	13 7	70	12 9
	021	4	2 5	30	5 5
	111	7	4 3	38	7 0
	201	9	5 6	26	4 8
	030	4	2 5	9	1 7
	120	3	1 9	7	1 3
	210	5	3 1	5	0 9
	300	12	7 5	5	0.9
	Number of cells	161	100 1	543	100 0
	N	96	19 9	192	11 8
	C	343	71 0	1238	76 0
	B	44	9 1	199	12 2
	Number of grains	483	100 0	1629	100 0

Table 5 shows the results of calculations using different μ-values, namely 0 5 – 1 0 – 1 5 – and 2 0 grains per cell corresponding to the lowest and highest background counts, respectively, encountered in the stripping emulsion used in this study For a mean background of 2 grains per cell, i e $\mu = 2$, which was observed in several cases (cf Table 6) the probability of 6 to 9 grains per cell is 3 65 per cent The probability of observing f ex 6 to 9 grains per cell varies considerably as a function of μ The chance of 10 grains per cell, or more, due to background is 1 per cent at most for all μ-values listed in the table

TABLE 7

Calculated Frequencies of (N) Grains per Cell due to Background and Observed Frequencies in 2 Animals Sacrificed 96 h post Injection (Background is Expressed in Grains per Mean Cell Area of 70 sq microns)

Number of grains (N)	Background = 2 175		Background = 0 834	
	Calc β_N	Obs h_N	Calc β_N	Obs h_N
0	0 163	0 230	0 462	0 280
1	0 249	0 256	0 331	0 254
2	0 227	0 170	0 142	0 192
3	0 160	0 114	0 047	0 096
4	0 097	0 106	0 014	0 050
5	0 053	0 042	0 003	0 040
6	0 0268		0 0008	
7	0 0128	0 048 (N = 6–9)	0 0002	
8	0 0058			0 088 (N = 6–10)
9	0 0025		0 0001 (N = 8–10)	
10	0 0011			
11	0 0005	0 034 (N = 10–12)		
12	0 0003			
	1 0000		1 0000	

Table 6 columns 5, 6 and 7 show the observed frequencies of cells with zero grains (h_0) for each autoradiogram studied and the frequencies (β_0) calculated on the assumption that the mean cell area viewed in the microscope is 47 sq micra (column 3) and 70 sq micra (column 4), respectively It can be seen that a mean cell area of 70 μ^2 gives the best agreement with the observed frequency of cells with zero grains

The decision how many grains per cell are required to denote a cell as "significantly labelled' depends on the contribution of the background to the total number of grains per cell This point is illustrated in Table 7 The mean number of background grains per cell of the autoradiograms of two animals sacrificed 96 h after injection of tritiated thymidine (Series II) was 2 175 and 0 834, respectively Column 2 of Table 7 shows the calculated frequencies of occurrence of zero – one – two – and up to nine grains per cell due to background and column 3 shows the observed frequencies On the autoradiogram with the high background, the calculated and the observed frequencies are similar

up to 9 grains per cell, and therefore 'significantly labelled cells" on this plate are those with 10 grains or more On the autoradiogram with the lower background, the observed frequency of 5 grains per cell is 10 times higher than that due to background, and 99 9 per cent of the [illegible]

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TABLF 8

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	C 001	469	60 5	1141	76 3
	B 010	114	14 7	167	11 2
		775	100 0	1496	100 1
S=2	002	179	51 7	509	65 3
	011	30	8 7	79	10 1
	020	18	5 2	38	4 9
	101	68	19 7	101	13 0
	110	23	6 6	21	2 7
	200	28	8 1	31	4 0
	Number of cells	346	100 0	779	99 9
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	012	6	3 7	55	10 1
	102	22	13 7	70	12 9
	021	4	2.5	30	5 5
	111	7	4 3	38	7 0
	201	9	5 6	26	4 8
	030	4	2 5	9	1 7
	120	3	1 9	7	1 3
	210	5	3 1	5	0 9
	300	12	7 5	5	0 9
	Number of cells	161	100 1	543	100 0
	N	96	19 9	192	11 8
	C	343	71 0	1238	76 0
	B	44	9 1	199	12 2
	Number of grains	483	100 0	1629	100 0

TABLE 8 (continued)

(1)	NCB (2)	Series I		Series II	
		Obs (3)	% (4)	Obs (5)	% (6)
S = 4	004	28	38.9	112	40.4
	013	2	2.8	19	6.9
	103	9	12.5	42	15.2
	022	2	2.8	18	6.5
	112	2	2.8	30	10.8
	202	9	12.5	17	6.1
	031	2	2.8	3	1.1
	121	2	2.8	4	1.4
	211	2	2.8	5	1.8
	301	2	2.8	10	3.6
	040	1	1.4	2	0.7
	130	1	1.4	4	1.4
	220	3	4.2	4	1.4
	310	2	2.8	4	1.4
	400	5	6.9	3	1.1
	Number of cells	72	100.2	277	99.8
	N	74	25.7	186	16.8
	C	179	62.2	783	70.7
	B	35	12.2	139	12.5
	Number of grains	288	100.1	1108	100.0

In all experiments discussed in this work, cells with 10 grains or more are significantly labelled, although a few of the grains may be due to random background.

The Attribution of Silver Grains to Different Cell Regions

In the tissue sections investigated, mast cells are readily recognized due to the metachromatic staining of the cytoplasmic granules. The nucleus frequently appears as a clear, faintly bluish zone. As viewed in the microscope, the area occupied by the cytoplasm varies greatly. Occasionally, it is almost 10 times the area of the nucleus. In the evaluation of the localization of the grains over different cell regions, a correction must be applied for the different contributions of the background to the blackenings observed in different cell regions.

Table 8 illustrates this point for cells with between one and four grains. Column 1 gives the number of grains per cell, and column 2 all possible placements of the given number of grains within the cell regions denoted as *N*ucleus, *C*ytoplasm and *B*oundary. Columns 3 and 4 present the number of cells observed with the grain localizations specified in column 2, in absolute numbers and in per cent. The horizontal rows NCB give the number of grains located over these regions in per cent of all grains observed.

It follows from this analysis that, up to 4 grains per cell, the localization of the grains over different cell regions is the same in all plates

TABLE 9

Regional Distribution of Silver Grains over Nucleus, Cytoplasm, and Boundary Zone of Mast Cells

Region	Series	Total number of grains observed over cells with (N) grains per cell (N) 1 – 4	5	6 – 9	10 –
	I	2238	180	332	782
	II	5791	810	1240	2821
	III	–	435	643	924
		Percentage distribution of grains over N C B			
N	I	22 7	20 0	25 3	25 1
	II	14 9	20 8	25 7	33 7
	III	–	19 3	17 4	29 4
C	I	64 7	70 6	63 0	60 7
	II	73 9	66 2	63 9	58 6
	III	–	75 4	75 1	60 9
B	I	12 6	9 4	11 7	14 2
	II	11 2	13 0	10 4	7 7
	III	–	5 3	7 5	9 7

TABLE 8 (continued)

(1)	NCB (2)	Series I Obs (3)	Series I % (4)	Series II Obs (5)	Series II % (6)
S = 4	004	28	38 9	112	40 4
	013	2	2 8	19	6 9
	103	9	12 5	42	15 2
	022	2	2 8	18	6 5
	112	2	2 8	30	10 8
	202	9	12 5	17	6 1
	031	2	2 8	3	1 1
	121	2	2 8	4	1 4
	211	2	2 8	5	1 8
	301	2	2 8	10	3 6
	040	1	1 4	2	0 7
	130	1	1 4	4	1 4
	220	3	4 2	4	1 4
	310	2	2 8	4	1 4
	400	5	6 9	3	1 1
	Number of cells	72	100 2	277	99 8
	N	74	25 7	186	16 8
	C	179	62 2	783	70 7
	B	35	12 2	139	12 5
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The Attribution of Silver Grains to Different Cell Regions

In the tissue sections investigated, mast cells are readily recognized due to the metachromatic staining of the cytoplasmic granules The nucleus frequently appears as a clear, faintly bluish zone As viewed in the microscope, the area occupied by the cytoplasm varies greatly Occasionally, it is almost 10 times the area of the nucleus In the evaluation of the localization of the grains over different cell regions, a correction must be applied for the different contributions of the background to the blackenings observed in different cell regions

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mast cells which incorporated tritiated thymidine in the first few hours after the injection The calculations presented in Table 10 were carried out in order to elucidate this problem Column 2 lists the average number of grains per cell derived by dividing in each case the total number of grains registered by the total number of cells examined Column 3 shows the net values after the background has been subtracted The variances of the distributions for the observed number of grains per cell and for the background respectively are given in columns 4 and 5 and the variances of the differences in column 6 The increase in these differences as a function of time is very marked indeed and provides evidence for the assumption that labelled cells have entered the cell population at a time when circulating tritiated thymidine was no longer available

CONCLUSIONS

These studies of connective tissue with intense regeneration activity of mast cells have shown that within 20 h after injection of tritiated thymidine at least 2 per cent of the metachromatically stained granulated mast cells have taken up thymidine which is understood to indicate mitotic activity About 96 h post injection however the number of significantly labelled mast cells had increased markedly

The experimental data indicate that a large fraction of the labelled mast cells observed between one and seven days post injection must have taken up the labelled DNA precursor at a time when they could not be identified as mast cells by available histological means In this latter stage they must be called fibroblast like cells or even fibroblasts Between 20 h and 96 h post injection these cells have developed into mast cells characterized by metachromatic cytoplasmic granules

The following conclusions may be drawn

1) Some connective tissue cells divide in a stage in which they fulfil all histological criteria of mast cells
2) The majority of mast cells however divide mitotically in the developmental phase of non granular fibroblast like precursor cells

These results offer an explanation for the prevailing confusion concerning mast cell and fibroblast function It can be understood now why especially in tissue culture some investigators find fibroblasts to be the *origin of* ground substance mucopolysaccharides while others ascribe this *function* to the mast cells Insufficient histological criteria are probably to blame for the disagreement

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1 *Downey H* The development of histogenous mast cells of adult guinea pig and cat and the structure of the histogenous mast cells of man Folia Haematol 16 49 1913

of Series I and II On the blank plate, 86 3 per cent of the grains were attributed to the cytoplasm, 7 3 per cent to the nucleus, and 6 4 per cent to the boundary zone The results of Table 8 are to be expected if the majority of the grains is due to random background

The observations made on cells with 5, 6 to 9, or more than 9 grains per cell are listed in Table 9 With increasing blackening, the localization of the grains changes in favour of the nucleus However, this effect is by no means spectacular, and it seems not to be markedly dependent on the time clapsed between injection of tritiated thymidine and sacrifice

As emphasized by *Maurer & Primbsch* (8), absorption and self- absorption of the beta-particles are functions of the absorber mass If it is assumed that the dry mass (expressed in mg/cm²) of the mast cell nucleus is greater than that of the cytoplasm, beta-particles originating from a labelled nucleus will be re-absorbed more strongly than those originating from the cytoplasm This phenomenon will introduce an additional systematic error into the grain patterns observed, giving rise to a levelling of the blackenings produced over the nucleus and the cytoplasm, respectively

TABLE 10

Time	Average no of grains cell		Variance of distribution acc to no of grains		
(1)	Obs (2)	Obs backgr (3)	Obs (4)	Backgr (5)	Diff (6)
0 5 h	0 605	–0 003	2 946	0 682	2 264
2	0 611	–0 268	6 341	1 172	5 170
12	0 950	0 492	5 730	0 500	5 230
16	1 133	0 868	5 418	0 279	5 139
20	0 686	–0 259	3 197	1 124	2 073
12	0 730	0 156	3 451	0 640	2 821
24	1 744	0 990	14 857	0 868	5 989
48	1 753	0 178	8 866	2 071	6 789
48	2 066	0 645	8 956	1 825	7 131
72	1 941	1 171	26 264	0 889	25 375
72	2 592	0 472	15 488	3 019	12 469
96	2 546	0 371	14 938	3 121	11 817
96	2 644	1 810	27 080	0 973	26 107
120	2 194	1 190	15 445	1 206	14 239

A small number of heavily labelled cells were observed which had a distinctly labelled nucleus and very few grains located over the cytoplasm The autoradiogram from the 7-day-animal offers the most striking example of a preponderance of mast cells with a labelled nucleus In many instances, however, the localization of the grains over "hot" cells could not be specified, either because grains occupied the whole cell area, or because the outline of the nucleus could not be recognized

The question arises whether the heavily labelled cells observed between four and seven days post injection belong to the same group of

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EXPERIMENTAL INVESTIGATION ON INFLUENCE OF OESTROGEN ON SPREAD OF TRANSPLANTED ROUS RAT SARCOMA

By

TOM SALDEEN

Received 22 x 64

The effect of cortisone on the spread of transplanted tumours has been the subject of many experimental investigations (*Agosin et al* 1952, *Baserga & Shubik* 1954, *Gasic & Gasic* 1957, *Ghose* 1958 *Iversen* 1957, *Lapis & Sagi* 1956 *Moore et al* 1960 *Pomeroy* 1954, *Saldeen* 1963, *Zeidman* 1962 and others) Other hormones have been studied less in this respect Only few papers deal with the effect of oestrogen on metastasization

Martin et al (1939) found no effect of oestrogen treatment on the spread of Brown Pearce epithelioma inoculated in one testis of rabbit

Schmähl & Rieseberg (1958) studied the influence of oestrogens (diaethyl stilbestrol) on the fate of intravenously injected Yoshida sarcoma and Walker carcinosarcoma cells in rats Ten of 28 oestrogen treated rats with Yoshida sarcoma and 1 of 8 with Walker carcinosarcoma developed metastases in the mammary glands while no such secondaries were seen in rats not treated with oestrogen In addition the proportion of takes by Yoshida sarcoma and Walker carcinosarcoma in oestrogen treated rats was 100 per cent against 93 per cent and 75 per cent respectively in the controls

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MATERIAL AND METHODS

Animals 192 white Sprague Dawley ... re fed on a standard diet consisting of co

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... r tissue was pressed through a steel sieve with 163 meshes/cm2 and suspended in Hank s solution with antibiotics The suspension was passed through a syringe fitted with a 22 gauge needle The tumour cells were counted in a Buerker haemocytometer using the eosin method (*Schrek* 1936)

Oestrogen Dimenformon® (Pharmacia) containing 2 5 mg of oestradiol benzoate per ml was used. The controls were given olive oil

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lungs on the 16th–33rd day after injection of the tumour cells (mean 24th day) (Table 2) Nine of the controls died on the 17th–37th day (mean 25th day), and had also widespread tumour nodules in the lungs No significant differences regarding number or size of the tumour nodules were seen between the two groups

Treatment with oestrogen thus did not enhance the *growth of the lung tumours*

TABLE 2

Number of Rats with Tumour Take in Lungs after Intravenous Injection of 1.5 × 10⁶ Tumour Cells in Oestrogen Treated Rats and Controls

Rat group	Oestradiol benzoate (mg)	Tumour take	Interval between injection and death in days (mean)
1	0 0025	10/10	24
2		9/10	25

Experiment 3

The material consisted of 40 rats They were divided into 4 equal groups and injected intramuscularly in the left thigh with 10^7 living tumour cells Group 4 (female spayed rats) were treated from 2 days before the injection of the tumour until the end of the experiment with daily i m injection of 0 25 mg of oestrodiol benzoate, while groups 1–3 (Table 3) were injected with olive oil

The size of the tumours in the different groups 1, 2, 3 and 4 weeks, respectively, after injection of the tumour cells is given in Table 3 The volumes of the tumours were calculated according to the formula $V = 0\ 5236\ d^3$, where d is the mean diameter of the tumour The mean diameter was calculated according to the formula $d = \sqrt{d^1 \times d^2}$ (d^1 and d^2 length and breadth of tumour) The rats were killed 4 weeks after injection It is clear from the table that at the end of the experiment the tumours in the oestrogen treated rats were smaller than those in the other groups (The difference was significant)

The groups *did not differ regarding the* histological appearance of the tumours

The spread of the tumours to the lymph nodes is given diagramatically in Fig 1 which shows that no significant differences were found between groups 1 3 In group 4, however, metastases particularly to the re[illegible]

sign[illegible]

mean[illegible]

of ly[illegible] of the tumour in the oestrogen treated rats often caused a marked enlargement of the retroperitoneal lymph nodes (Fig 2) Seven of the oestrogen treated rats had small pulmonary metastases while no lung nodules were seen in other rats Pulmonary metastases were seen only in animals with secondary growths in the

RESULTS

Experiment 1.

52 rats were inoculated intravenously into one of the tail veins with 10^6 living tumour cells They were divided into 4 groups (Table 1) Groups 1 and 2 contained each 16 spayed female rats All rats were spayed 14 days before the start of the tumour experiment Group 1 was treated with a daily intramuscular injection of 0 25 mg of oestradiol benzoate The first injection was given 2 days before inoculation with the tumour Groups 2, 3 and 4 were controls Groups 3 and 4 consisted of 10 non-spayed female and 10 male rats, respectively

TABLE 1

Number of Rats with Tumour Take in Lungs after Intravenous Injection of 10^6 Tumour Cells in Oestrogen Treated Rats and Controls

Rat group	Sex	Spayed	Oestradiol benzoate (mg)	Tumour take	Interval between injection and death in days (mean)
1	♀	+	0 25	13/16	24
2	♀	+		4/16	22
3	♀	—		2/10	22
4	♂	—		2/10	22

Thirteen rats in group 1 died on the 16th–45th day after the injection of the tumour cells (mean 24th day) Four rats in group 2 died on the 10th–25th day (mean 22nd day) Two rats in group 3, and 2 in group 4, died on the 16th–25th day (mean 22nd) All rats that died had numerous tumour nodules in the lungs A few also had tumour in the hilar and mediastinal lymph nodes, kidneys or adrenals In none of the rats were metastates seen in the mammary glands The other rats were killed on the 90th day They showed no signs of tumour growth

Treatment with large doses of oestrogens thus resulted in a higher frequency of tumour takes in the lungs than in untreated control rats inoculated with the same tumour dose The difference was significant No significant differences were seen between males and females or between spayed and non-spayed females

Experiment 2

20 spayed female rats were inoculated intravenously with 1 5 × 10^6 tumour cells This tumour dose was supposed to give takes also in the controls Ten of the rats were treated with 0 0025 mg of oestradiol benzoate i m daily from 2 days before the injection of the tumour and throughout the experiment This dose of oestrogen was used since larger doses often give an inhibition of tumour growth The other rats were controls

All oestrogen treated rats died with numerous tumour nodules in the

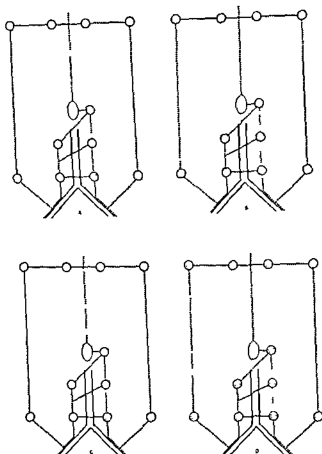


Fig 1

Number of rats with lymph node metastases The rats had intramuscular tumours in the left thigh The four groups contained 10 rats each

A Female intact untreated rats
B Male intact untreated rats
C Female spayed untreated rats
D Female spayed rats treated with 0 25 mg of oestradiol benzoate daily

(Names of the various groups of lymph nodes are given in Fig 4)

cisternal lymph nodes, so that spread to the lungs might have been lymphogenic (*Saldeen* 1963)

Experiment 4

In another experiment 30 spayed female rats were used They were divided into five equal groups All rats were inoculated intramuscularly in the left thigh with 10 living tumour cells

From 2 days before the injection of the tumour until the end of the

TABLE 3

Tumour Volume and Carcass Weight (without Tumours) of Rats with Tumours in the Left Thigh and Treated with Oestrogen

Rat group	Sex	Spayed	Oestradiol benzoate (mg)	Carcass (g)		Tumour volume (cm³)							
						7 days		14 days		21 days		28 days	
					s		s		s		s		s
1	♀	—		143 ± 5	9	7 59 ± 1 09	3 32	32 0 ± 9 33	3 46	43 8 ± 5 57	13 7	44 4 ± 7 42	16 5
2	♂	—		133 ± 10	27	9 53 ± 0 86	2 44	40 9 ± 5 10	14 5	55 2 ± 8 43	18 8	50 5 ± 13 5	26 9
3	♀	+		151 ± 16	35	8 13 ± 0 60	1 89	32 8 ± 3 46	10 5	39 5 ± 4 00	9 85	40 8 ± 7 42	14 9
4	♀	+	0 25	147 ± 9	25	6 79 ± 1 46	4 58	24 1 ± 3 09	9 27	38 7 ± 6 63	17 5	21 2 ± 4 00	9 06

TABLE 4

Carcass Weight (without Tumours), Tumour Volume, Weight of Adrenals and Anterior Lobe of Hypophysis in Rats with Tumours in the Left Thigh and Treated with Various Doses of Oestrogen

Rat group	Oestradiol Benzoate (mg)	Carcass (g)	s	Adrenals (g)	s	Hypophysis (g)	s	Tumour volume (cm^3) 7 days	s	14 days	s	21 days	s
1		188 ± 8	19	0 078 ± 0 006	0 014	0 007 ± 0 00005	0 0004	10 5 ± 1 04	2 55	34 0 ± 2 96	7 28	62 1 ± 7 48	18 4
2	0 25	215 ± 7	15	0 080 ± 0 004	0 008	0 016 ± 0 0004	0 0009	8 05 ± 0 96	0 40	20 2 ± 1 55	3 74	31 2 ± 1 95	4 3
3	0 025	208 ± 6	14	0 071 ± 0 005	0 012	0 017 ± 0 0002	0 001	8 39 ± 0 49	1 19	18 0 ± 1 80	4 36	22 2 ± 4 36	9 80
4	0 0025	215 ± 16	28	0 103 ± 0 010	0 019	0 012 ± 0 0001	0 0007	12 5 ± 1 71	4 24	33 2 ± 6 78	15 1	44 4 ± 6 93	12 0
5	0 00025	213 ± 3	7	0 086 ± 0 006	0 016	0 010 ± 0 00007	0 0006	11 3 ± 0 57	1 29	38 2 ± 5 39	12 0	34 4 ± 6 93	12 0

TABLE 5

Carcass Weight (without Tumours), Tumour Volume, Weight of Adrenals, Anterior Lobe of Hypophysis and Spleen in Rats with Intramuscular Tumours (Left Thigh) and Treated with Various Doses of Oestrogen

Rat group	Oestradiol Benzoate (mg)	Carcass (g)	s	Tumour (cm^3)	s	Adrenals (g)	s	Hypophysis (g)	s	Spleen (g)	s
1		200 ± 7	21	62 2 ± 5 4	13 6	0 072 ± 0 006	0 010	0 007 ± 0 00006	0 0006	1 210 ± 0 210	0 531
2	0 0025	193 ± 8	22	39 3 ± 5 0	13 5	0 066 ± 0 004	0 016	0 008 ± 0 00007	0 0005	0 762 ± 0 136	0 361
3	0 025	163 ± 11	13	43 1 ± 7 4	15 6	0 078 ± 0 007	0 017	0 011 ± 0 0001	0 0008	0 793 ± 0 060	0 097
4	0 25	190 ± 8	21	40 6 ± 4 1	12 6	0 080 ± 0 005	0 013	0 013 ± 0 00002	0 001	0 867 ± 0 069	0 136
5	1 0	169 ± 9	10	22 2 ± 3 8	8 7	0 080 ± 0 005	0 015	0 012 ± 0 00003	0 0007	0 665 ± 0 073	0 159



Fig 2

Rat treated with oestradiol benzoate Tumour intramuscularly in the left thigh Note large metastases in left lumbar left renal and cisternal lymph nodes

experiment groups 2–5 were treated daily with oestrogen in different concentrations (Table 4) Group 1 served as control

The sizes of the tumours in the thigh 1 2 and 3 weeks respectively after the transplantation are given in Table 4 The weight of the animals the adrenals and the pituitary anterior lobes are also given in the table

The rats were killed after 3 weeks The oestrogen treated rats then had significantly smaller tumours than the controls rats The anterior pituitary glands were as a rule larger in the oestrogen treated rats than in the controls Histologically no differences were seen between the primary tumours in the various groups

The spread to the lymph nodes is given diagrammatically in Fig 3

No significant differences were seen between the various oestrogen treated groups regarding the lymph node metastases All oestrogen treated rats taken together however had metastases more often in the left renal nodes (the difference was significant) and in the cisternal nodes (the difference almost significant) than the controls In addition the lymph node metastases in the oestrogen treated rats were often somewhat larger than those in the controls

Experiment 5

50 intact female rats were used They were divided into 5 equal groups and inoculated intramuscularly with 10 living tumours cells Groups 2 5 were treated with oestradiol benzoate i m in varying doses (Table 5) from 2 days before the tumour inoculation and throughout the experiment Group 1 was control The rats were killed after 4 weeks The weights of the animals adrenals spleen anterior pituitary gland and the final size of the tumours are given in Table 5

The thigh tumours and the spleen were smaller and the adenohypophysis larger in the oestrogen treated rats than in the controls

Histologically there were no differences between the thigh tumours spleen or adrenals in the various groups The adenohypophysis of the oestrogen treated rats showed an increased number of chromophobe cells and degranulated basophile and sometimes acidophile cells

The spread of the tumours is given diagramatically in Fig 4 Oestrogen treated rats had more often lymph node metastases than the controls especially to the remote lymph nodes Eight of the oestrogen treated rats but none of the controls had pulmonary metastases The effect of oestrogen on lymphatic tumour spread did not increase significantly with larger doses of oestrogen

DISCUSSION

It is clear from the experiments described above that treatment of the rats with oestradiol benzoate increased the lymphatic spread of the Rous rat sarcoma transplanted intramuscularly The effect on the tumour spread may occur in one or both of the two stages of metastasization

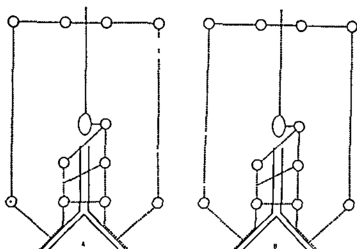
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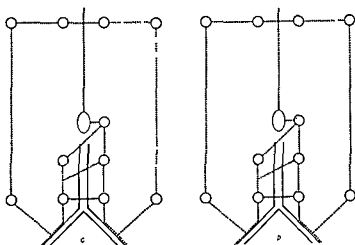
Fig 3

Number of rats with lymph node metastases The rats had intramuscular tumours in the left thigh The five groups consisted of 6 spayed female rats each

A Untreated rats
B Rats [illegible]

(Na[illegible] various groups of lymph nodes are given in Fig 4)





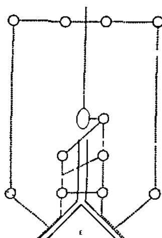


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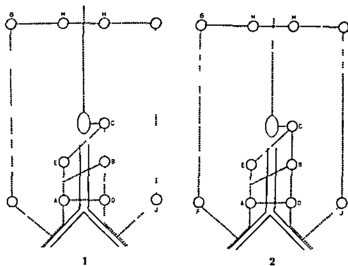
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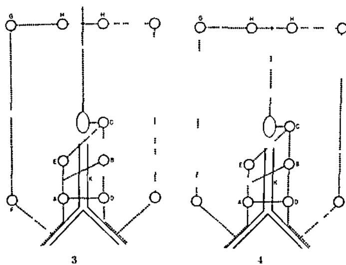
Fig 3

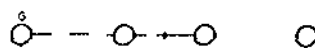
Number of rats with lymph node metastases The rats had intramuscular tumours in the left thigh The five groups consisted of 6 spayed female rats each

A Untreated rats
B Rats treated with 0.25 mg oestradiol benzoate daily
C Rats treated with 0 025 mg oestradiol benzoate daily
D Rats treated with 0 0025 mg oestradiol benzoate daily
E Rats treated with 0 00025 mg oestradiol benzoate daily

(Names of the various groups of lymph nodes are given in Fig 4)







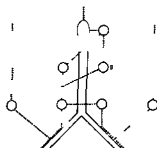


Fig 4

1) Release of the tumour cells from the primary tumour and their invasion of contiguous tissue
2) Deposition and growth of the tumour cells in the lymph nodes

Influence on the first stage seems less likely The tumours in the oestrogen treated rats were smaller and showed a slower increase in size than the tumours in the controls Histologically, there were no differences between the primary tumours of the two groups No disintegration of the stroma with loosening of the tumour cells occurring in cortisone treated rats (*Saldeen* 1963) was seen

It is possible that the oestrogen treatment renders the lymph nodes more susceptible to the tumour cells in the same way as it increases the frequency of pulmonary takes after intravenous injection of tumours cells But the present investigation offers no explanation for the mechanism of such an increase in susceptibility The oestrogen may have a *direct* effect on the lymph nodes *e g* on their immunologic or phagocytic activity or an *indirect* effect via *e g* the anterior hypophysis which showed pronounced changes in the oestrogen treated rats

Guimarães et al (1963) fell that the sex hormones ' exert their action through some modifying effect on endothelial adherence and blood coagulability These factors are probably of importance in haematogenic but not in lymphogenic, tumour spread

Although no differences existed between male and female or between spayed and unspayed female rats both small and large doses of oestrogen promoted tumour spread The effect did not increase significantly with larger doses of oestrogen

SUMMARY

Treatment with oestradiol benzoate increased the lymph node metastasization in rats with Rous rat sarcoma transplanted intramuscularly and also increased the frequency of pulmonary takes after intravenous injection of Rous rat sarcoma cells

---

Fig 4

Number of rats with lymph node metastases *The rats had intramuscular tumours in the left thigh* The five groups consisted of 10 female intact rats each

1 Untreated rats
2 Rats treated with 0 0025 mg oestradiol benzoate daily
3 Rats treated with 0 025 mg oestradiol benzoate daily
4 Rats treated with 0 25 mg oestradiol benzoate daily
5 Rats treated with 1 0 mg oestradiol benzoate daily

Names of the groups of lymph nodes

A Right lumbar
B Left renal
C Cisternal
D Left lumbar
E Right renal
F Right inguinal
G Right axillary
H Mediastinal
I Left axillary
J Left inguinal

In spread of the transplanted Rous rat sarcoma no differences was seen between untreated male and female rats or between spayed and unspayed female rats

The mechanism of the oestrogen effect is briefly discussed

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EXPERIMENTAL INVESTIGATION ON INFLUENCE OF HYALURONIDASE ON SPREAD OF TRANSPLANTED TUMOURS

By

TOM SALDEEN

Received 22 x 64

Hyaluronidase has been shown to promote tumour spread (*Gopal Ayengar & Simpson* 1947, *Russo & Terranova* 1953) It increased the extent of tumour growth in the mediastinal lymph nodes in rats with intraperitoneal Rous rat sarcoma whereas treatment of the rats with a hyaluronidase inhibitor (polyphloretin phosphate) diminished the spread of the tumour to these nodes (*Saldeen* 1963)

In preliminary experiments the author has found that intense treatment of the rats with hyaluronidase before infiltrative growth of the transplanted sarcoma has started also results in increased tumour spread to the mediastinal lymph nodes The hyaluronidase inhibitor decreased the number of viable tumour cells in the lymph nodes and reduced the resorption of red blood corpuscles from the peritoneal cavity

The results of these experiments suggest that hyaluronidase increases the permeability of the peritoneal membrane and thereby facilitates the passage of cells from the peritoneal cavity to the lymphatic system

The present investigation is concerned with the effect of hyaluronidase on the occurrence of viable tumour cells *in the lymph from the thoracic duct* in rats with intraperitoneal tumours

MATERIAL AND METHODS

Animals

Female white rats of the Sprague Dawley strain weighing about 200 g were used They were fed on a standard diet consisting of commercial rat pellets and water ad libitum

The mice were of a strain bred for many years in this laboratory as a closed colony

Tumours

tu [illegible]
di [illegible]
Sc [illegible] and maintained by serial subcutaneous transfer in new born rats

Ehrlich mouse ascites carcinoma The ascites carcinoma used was of the nearly tetraploid type It has been maintained by serial transplantation in adult mice

Preparation of Tumour Suspension

The technique used is a slight modification of a method described by *Madden & Burk* (1961) The rat tumour was first weighed cut into small pieces with scissors and transferred to an Erlenmeyer flask Five cc of 0 25 per cent crude trypsin in a modified Earle s solution (*Madden & Burk*) and 0 2 cc of an 0 04 per cent DNAase solution per g of tumour tissue were added The mixture was then agitated for 1 hour on a magnetic stirrer and the supernatant was decanted through gauze and centrifuged at 300 r p m for 10 minutes in an MSE Super minor centrifuge The cells were resuspended in modified Earle's solution containing a drop of DNAase and counted in a Buerker haemocytometer, using the eosin method described by *Schrek* (1936) Only uncoloured cells (living) were counted

In comparison with the mechanical techniques for tumour cell separation the enzymatic method gave a higher percentage of single and non eosin stained cells

Determination of Tumour Cells in the Thoracic Duct

The thoracic duct was catheterized in the neck by a method described previously (*Saldeen & Linder* 1960) Lymph was collected in sterile glass flasks containing 1 ml of a solution of 200 μg penicillin 200 μg streptomycin and 25 U of heparin per ml The temperature of the glass flasks was maintained at 4° C. The lymph was centrifuged for 15 minutes at 2 000 r p m One ml of the sediment was injected intraperitoneally into new born rats (R R sarcoma) or adult mice (Ehrlich mouse ascites carcinoma) Intraperitoneal injection was used since fewer cells are needed for tumour take by this route than by *e g* the subcutaneous The exact time of tumour take is however somewhat easier to determine by the subcutaneous method

In some experiments the lymph was filtered on a Millipore filter and the tumour cells were counted (*Saldeen* 1963)

Pharmacological Preparations

Hyaluronidase Hyalas® (Leo) was used Each rat received 200 I U in 1 ml of physiological saline intraperitoneally every 6th hour the controls saline alone

RESULTS

Sixteen rats were injected intraperitoneally with 10^8 living tumour cells from *Ehrlich mouse ascites carcinoma* On the 1st day after the injection 8 of the rats were treated with hyaluronidase, the other were *controls* The thoracic duct was catheterized in all animals on that day and the lymph analysed for viable tumour cells Five of the mice injected with lymph from the hyaluronidase-treated animals developed tumours within a mean period of 18 days Of the mice injected with lymph from the controls 3 developed tumours after a mean latency of

TABLE 1

Interval (Days) between Injection of Lymph and Tumour Take (Development of Ascites)

Rat group	Tumours: Ehrlich					Tumours: Rous			
Hyalas treated	16	17	17	17	23	12	12	13	15
Controls	17	18	24			13	13	16	

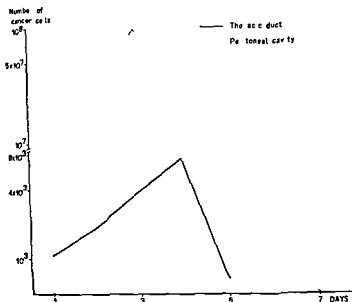


Fig 1

Number of tumour cells in the thoracic duct in one rat with intraperitoneally Ehr lich mouse ascites carcinoma compared with the average number of tumour cells in the peritoneal cavity in 4 rats after injection of the same tumour dose (10^7 tumour cells)

20 days (Table 1) The difference was not significant The other mice developed no tumour within 3 months

During this experiment some rats were catheterized for periods up to 10 days Tumour cells could not be demonstrated in the lymph after the 3rd day except in one rat (not treated with hyaluronidase) in which tumour cells were found daily during the first 5 days The number of cancer cells in the lymph was per day 1 088 1 872 3 780 7 625 and 689 respectively (Fig 1) The number was proportional to the average number of tumour cells seen in the peritoneal cavity on the corresponding days in 4 other rats after injection of the same tumour dose (Fig 1)

The lymph tap seemed to influence the growth of the tumour Of 6 rats catheterized for 7 days or more 4 had still tumour in the peritoneal cavity 10 days after the tumour injection (Table 2) In control animals (not catheterized) the tumour soon regressed and after 7 days no tumour cells could be demonstrated in the peritoneal cavity The catheterized animals had smaller spleens and adrenals and a reduced number of lymphocytes in the lymph (Table 3 and 4) In the catheterized rats the adrenal cortex showed a reduced lipid content and the spleen exhibited atrophy of the white pulp more pronounced in rats catheterized for 7 days than in those catheterized for 3 days

In the experiments with *Rous rat sarcoma* the rats were catheterized on the 11th day after the tumour injection

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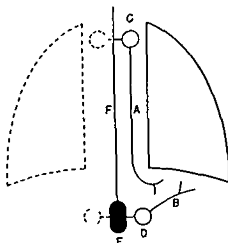


Fig 2

Diagrammatic presentation of the main lymphatic pathways from the peritoneal cavity

A Parasternal lymph vessels
B Retroperitoneal lymph vessels
C Mediastinal lymph nodes
D Cisternal lymph nodes
E Cisterna chyli
F Thoracic duct

DISCUSSION

Intense treatment with hyaluronidase increases the permeability of the peritoneum in the rabbit (*Fries* 1956)

It also promotes the tumour spread from the peritoneal cavity to the regional lymph nodes in rats (*Saldeen* 1963)

In the present investigation, however, hyaluronidase did not increase the number of tumour cells in the lymph of the thoracic duct in rats with intraperitoneal tumours

This discrepancy may be due to the fact that tumour cells probably have to pass through the lymph nodes before entering the thoracic duct (Fig 2) Though the hyaluronidase increases the permeability of the peritoneal membrane, it need not have this effect on the lymph node barrier

It is probable that the lymph node barrier is responsible for the non-occurrence of tumour cells in the thoracic duct after the 3rd day in all but one of the rats with intraperitoneal Ehrlich mouse ascites carcinoma In this exceptional rat there may have been a passage of cells via lymphatics running from the peritoneal cavity directly to the thoracic duct without passing through the lymph nodes Such anatomical abnormalities are sometimes seen (*Saldeen* 1963)

The present investigation provides no explanation of the mechanism of the improvement of the heterologous tumour growth during long

TABI E 2

Influence o' ' ~ Growth

The Rats jection

Days	Ascites (ml)	Tumour cells ml ascites × 10^6
7	1 3	56
7	2 5	167
10	0 7	132
10	3 5	43

TABLE 3

Influence of Lymph Collection on Weight (mg/g Body Weight) of Spleen and Adrenals

Days	Number of rats	Lymph volume (ml)	Spleen	Adrenals
0	6	0	6 1	0 5
3	3	146	4 2	0 5
7	3	373	4 6	0 3
10	4	576	4 2	0 4
28*	1	2416	1 1	0 2

* non-tumour rat (*Saldeen & Linder* 1960)

TABLE 4

Influence of Lymph Collection on Number of Lymphocytes in Thoracic Duct Lymph

Lymph volume (ml)	Number of rats	Lymphocytes mm³
0	3	36 300
10	1	22 300
35	1	20 400
60	1	13 100
85	1	10 800
105	1	6 000

Fourteen rats were injected intraperitoneally with 10^8 living R R sarcoma cells On the 11th day after the injection, when all rats had marked ascites, 7 of the rats were treated with hyaluronidase The thoracic duct was catheterized and the lymph analysed for viable tumour cells Four of the new born rats injected with lymph from the hyaluronidase-treated rats developed tumours after a mean latency of 13 days Of the rats injected with lymph from the controls 3 developed tumours within a mean period of 14 days (Table 1) The difference was not significant The other rats developed no tumour within 3 months

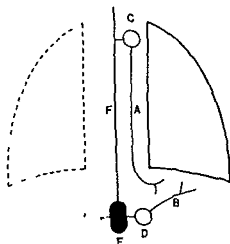


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THE FINE STRUCTURE OF THE SPINDLE CELL IN KAPOSI'S SARCOMA

By

MIKKO NIEMI and K. K. MUSTAKALLIO

Received 14 x 64

The number of investigations concerning the histology and histogenesis of the Kaposi's sarcoma has been rapidly increasing during the past four or five years (*Davies* 1963). Many of the modern laboratory techniques have been applied to exploit the origin of the neoplastic cells of Kaposi's sarcoma However, the results obtained by histochemical staining methods (*Pepler* 1959, *Dorfman* 1962, *Mustakallio et al* 1963, *Niemi et al* 1964) and by tissue culture techniques (*Cook* 1962, *Spence* 1962) have been controversial The nature and origin of the spindle or ovoid shaped tumor cell of Kaposi's sarcoma have therefore remained largely obscure

Electron microscopy has been also used to investigate the neoplastic lesion of Kaposi's sarcoma (*Pepler & Theron* 1962, *Yodaiken* 1962) Although these investigations have not been able to state definitely the cell of origin of this neoplasm, they have gathered evidence for the characteristic spindle shaped cells being modified Schwann cells This concept fits ho

was undertaken in order to gain new information of the fine structure of the spindle cell of Kaposi's sarcoma When describing the results special reference will be made to the age and stage of the lesions

MATERIAL AND METHODS

The m

... described elsewhere (*Niemi et al* 1964) ... of these patients have

The authors are grateful to Mr *M Nyholm* M Sc of the Electron Microscope Laboratory University of Helsinki for technical assistance

This study has been supported by grants from the *Sigrid Juselius Foundation* Helsinki *President J K Paasikivi Foundation for Cancer Research*, and from the *Finnish Medical Research Council*

term collection of lymph However, the lymphocyte tap may play a rôle, *e g* by reducing antibody production

SUMMARY

Intense treatment with hyaluronidase did not influence the number of viable tumour cells in the thoracic duct in rats with intraperitoneal Rous rat sarcoma or Ehrlich mouse ascites carcinoma

Long term collection of lymph enchanced the tumour growth in rats with intraperitoneal Ehrlich mouse ascites carcinoma

The results are briefly discussed

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Received 14 x 64

The number of investigations concerning the histology and histogenesis of the Kaposi's sarcoma has been rapidly increasing during the past four or five years (*Davies* 1963) Many of the modern laboratory techniques have been applied to exploit the origin of the neoplastic cells of Kaposi's sarcoma However, the results obtained by histochemical staining methods (*Pepler* 1959, *Dorfman* 1962, *Mustakallio et al* 1963 *Niemi et al* 1964) and by tissue culture techniques (*Cook* 1962, *Spence* 1962) have been controversial The nature and origin of the spindle or ovoid shaped tumor cell of Kaposi's sarcoma have therefore remained largely obscure

Electron microscopy has been also used to investigate the neoplastic lesion of Kaposi's sarcoma (*Pepler & Theron* 1962, *Yodaiken* 1962) Although these investigations have not been able to state definitely the cell of origin of this neoplasm, they have gathered evidence for the characteristic spindle shaped cells being modified Schwann cells This concept fits, however, poorly with the results of histochemical investigations (*Becker* 1962, *Dorfman* 1962, *Niemi et al* 1964) It is thus obvious that more work is necessary to clarify the picture The present study was undertaken in order to gain new information of the fine structure of the spindle cell of Kaposi's sarcoma When describing the results special reference will be made to the age and stage of the lesions

MATERIAL AND METHODS

The material consists of three tumors which were removed from two different patients Two of the lesions represented the angiosarcomatous stage of the disease third being an
devel sion representing the end stage of the
been he case reports of these patients have

The authors are grateful to Mr M Nyholm M Sc of the Electron Microscope Laboratory University of Helsinki for technical assistance

This study has been supported by grants from the *Sigrid Juselius Foundation* Helsinki President J K *Paasikivi Foundation for Cancer Research* and from the Finnish *Medical Research Council*

Small pieces of excised tumor tissue were fixed by immersion into ice cold 1 per cent osmium tetroxide solution buffered at pH 7 4 for periods between 2 and 3 hours The specimens were rapidly dehydrated and embedded in an epoxy resin (Epon 812) Thin sections were cut with a Porter Blum ultramicrotome using glass knives They were counterstained either with uranyl acetate or with lead hydroxide (*Watson* 1958), and examined in a Siemens Elmiskop I electron microscope

RESULTS

In the electron micrographs of the lesions several cell types could be identified Besides the characteristic spindle or ovoid shaped tumor cells, roundish or stellate cells were always present which were indistinguishable from normal histiocytes Red blood corpuscles were mostly numerous, and both endothelial cells and plasma cells could occasionally be identified The relative number of different cell types varied considerably depending on the duration of the disease In the early angiomatous lesions the extravasated erythrocytes were numerous, but they were mostly lying extracellularly in the vascular slits Erythrocytes were usually in close contact with spindle or ovoid shaped tumor cells In the old fibromatous lesion, the number of extravasated erythrocytes was small, the main cell type being the characteristic spindle cell, which were interspaced with collagen fibers

The most characteristic neoplastic cell was spindle or ovoid in its shape and had a large ovoid nucleus The latter contained a prominent nucleolus and its chromatin was usually evenly distributed (Fig 3) The cytoplasm contained moderately developed endoplastic reticulum, whose sacs were characteristically dilated (Fig 4) The number of mitochondria was small While many typical spindle cells were present in the old sarcomatous lesion, in the younger angiomatous lesions the tumor cells were more round and resembled in their ultrastructure normal histiocytes In fact, no clear-cut difference could be noticed between these two cell types, the transitional forms being quite frequent During the transition of a roundish histiocyte to a spindle cell a decrease in the amount of its cytoplasmic organelles took place simultaneously as the cell and its nucleus acquired the more ovoid or spindle shape Both the histiocyte-type and the spindle-type tumor cells contained often ingested hemosiderin pigment (Fig 4), which was, however, more abundant in the former type A characteristic feature for both cell types was the presence of phagocytosed erythrocytes in their cytoplasm (Fig 1) In the young lesions the erythrocytes were more often found lying inside the cytoplasm of the tumor cells which were mostly of the histiocyte-type, but in the old lesions only multilayered myelin-like figures could be seen (Figs 2, 3, 5 and 6) The transformation of the histiocyte-type cells to the spindle type was noticed to accompany the intracellular destruction of the erythrocytes



F g 1

In the l er part of the picture the nucleus of a hist ocyte type tumor cell is v s ble The cell has phagocytosed an erythrocyte × 23800

2

3



Fig 4

Two aggregations of electron dense granules (haemosiderin) within the tumor cell cytoplasm Note the distension of the ergastoplasmic sacs × 35000

Figs 2 3

Fig 2 In the cytoplasm of a tumor cell a multilamellar body is visible In the upper left corner of the picture an erythrocyte is lying in a vascular slit × 35000

Fig 3 A typical spindle shaped nucleus of a tumor cell In the cytoplasm of the neighbour cell several lamellar structures can be seen × 23800



Figs 5–6

Fig 5 Several erythrocytes phagocytosed by the same tumor cell and undergoing destruction The folding of their plasma membrane is extensive and there is some haemosiderin granules present amongst the membranous structures × 23800

Fig 6 Typical myelin figures within the tumor cell cytoplasm × 35000

DISCUSSION

Although most investigators agree that the spindle cell is the characteristic neoplastic cell in Kaposi's sarcoma (*Ackerman* 1962), there is no general agreement on the origin of this cell Most of the mesenchymal

The enzymatic histochemical staining properties of the spindle cells (*Mustakallio et al* 1963, *Niemi et al* 1964) are compatible with a reticuloendothelial function This together with the lack of staining characteristics of the neural tissue has suggested that the spindle cell of Kaposi's sarcoma represents a multipotent mesenchymal cell which, at different stages of the disease, plays a role of a phagocyte or a fibrocyte The findings presented in this paper support this hypothesis The main observation was a pronounced erythrophagocytosis by the histiocyte system of the lesions It was noticed that in the young lesions free erythrocytes in the vascular slits were numerous and many of them

were phagocytosed by the perivascular histiocytic cells In the old lesions however, the typical spindle shaped tumor cells were most frequently observed and their cytoplasma contained often multilayered bodies with myelin like configuration These were considered as being results of the breakdown of the ingested erythrocytes Similar intra cellular figures have been observed by *Stoeckenius* (1957) in the macrophages of the spleen during erythrophagocytosis Cephalin has been shown to be the main lipid component of the erythrocytes (*Ponder* 1948) and this compound can produce *in vitro* easily myelin like multi layered figures (*Baer et al* 1941)

Earlier electron microscopists have noticed the presence of intracytoplasmic bodies surrounded by two or more layers of a unit membrane (*Pepler & Theron* 1962) These structures have been taken as indicative for a Schwann cell origin of the spindle cells In our electron micro graphs two of the characteristics of a Schwann cell were constantly lacking we could not demonstrate the presence of a basement mem brane neither could we see any mesaxon connecting the multilayered bodies to the plasma membrane Therefore it seems unlikely that these cytoplasmic figures are true myelin of nerve fibers but they may be late results of erythrophagocytosis in the spindle cells of Kaposi s sarcoma

The cell of origin of the spindle cell in Kaposi s sarcoma has been exceptionally difficult to prove This seems to relay at least partly on too strong adherence to a concept that the different perivascular cells would be separate and identifiable entities However several workers have pointed out that cells of various origin can have a histiocytic *i e* phagocytic state (*Thomas* 1938 *Chevremont* 1942 *Policard* 1957) Some of the recent observations also support the view that even well dif ferentiated cell types can have similar functional stages when they act as histiocytes *i e* *Nusser* (1964) has demonstrated that the Schwann cell resembles a macrophage during Wallerian degeneration

The submicroscopic observations presented in this paper suggest that the roundish or oval cells which are indistinguishable from normal macrophages and are situated around the vascular slits of the young lesions of Kaposi s sarcoma and the typical spindle shaped tumor cells of the late stages of the disease are the same cell in different functional stages This cell thus corresponds to a multipotent mesenchymal cell Its development to a typical neoplastic spindle cell would be directed by the erythrophagocytosis and the late evolution of the tumor cell transforms it into a fibroblast We think that this cell belongs to the adventitial mesenchymal cells s c pericytes of the blood vessels whose endothelial budding gives *rise* to the extravasation of erythrocytes This process sets about an erythrophagocytosis which gradually leads to [illegible]
form[illegible]

Finally it should be mentioned that Baker et al (1961) have shown that repeated large subcutaneous doses of iron dextran can cause a gradual change of macrophages to spindle cells and lead to the development of collagen and to a sarcomatous change

SUMMARY

Three neoplastic skin lesions of histologically typical Kaposi s sarcoma were examined electron microscopically In the early angiomatous stage of the disease the tumor cells were of a histiocyte type and surrounded by numerous extravasated erythrocytes Some of these were undergoing phagocytosis by the perivascularly located tumor cells The erythrophagocytosis was accompanied by a gradual change of the histiocyte-type cells to the typical spindle-shaped tumor cell of the Kaposi s sarcoma In the latter stage the amount of endoplasmic reticulum and the number of mitochondria was markedly reduced while the nucleus was changed to oval in shape and contained a prominent nucleolus The cytoplasm of the spindle cells contained frequently multilayered myelin figures which were considered as being derived from the lipids of the ingested erythrocytes

The ultrastructure of the spindle cells of Kaposi s sarcoma did not give evidence for their origin from the Schwann cells but suggest rather a multipotent perivascular mesenchymal cell (pericyte) for the cell of origin of this disorder

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LIPOMELANOTIC RETICULOSIS, DIAGNOSED IN STERNAL MARROW

One Case

By

K Bak Pedersen

Received 18 x 64

During the past 30 years a number of reports have appeared, primarily in the dermatological literature, concerning superficial lymph node enlargement in connection with certain skin diseases, in particular diseases of an erythematous and pruriginous nature Microscopic study of these lymph nodes revealed characteristic lesions, the most important of which were proliferation of reticulum cells with deposits of fat and melanin and eosinophilia

Previous studies on lipomelanotic reticulosis were mainly concentrated on the macroscopic and microscopic appearance of the lymph nodes, on the relation of the disorder to certain skin diseases, and on the question as to whether the disorder was of a benign nature or a precursor of a malignant disease Furthermore, the difficulties in connection with the differential diagnosis which may arise out of the lymph node enlargement were often discussed

In 1892 *Jadassohn* (9, 10) reported a patient with lymph node changes in connection with pityriasis rubra Hebra In the periphery of the lymph nodes he found cells with a pigment, which showed a negative reaction when examined for iron content, but which could be bleached by means of hydrogen peroxide In another patient with an eczematous disorder he found also pigment deposits in the enlarged lymph nodes This pigment exhibited a positive iron reaction It is probable that the changes observed by Jadassohn were of the same nature as those described in detail by *Pautrier & Woringer* 40 years later.

Pautrier & Woringer (23, 24) described 11 patients, all of whom had for shorter or longer periods suffered from skin disorders, in most cases accompanied by erythema and pruritus Furthermore they had a moderate superficiel enlargement of the lymph nodes, either localized

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or generalized depending on the spread of the skin disorder Microscopy of the lymph nodes revealed a normal capsule with no signs of periadenitis By and large their structure was preserved showing distinct and normal lymph follicles However, the latter may be displaced to a certain extent because of reticulum cell proliferation, which was the most conspicious and dominant change The reticulum cells were large mononuclear and with effaced borders The cytoplasm was abundant and pale often vacuolated Another important change was a high content of fat and melanin in these cells Furthermore eosinophilia was always present on rare occasions also plasma cells were found *Pautrier & Woringer* suggested that the changes be termed "La reticulose lipomelanique" Later Hurwitt described this term as inaccurate and misleading stating that it did not appear clearly that the skin disorder was the primary disease and produced the changes in the lymph nodes Furthermore he pointed out that in some cases there were no deposits of fat and melanin Instead he suggested the term "Dermatopathic lymphadenitis

In his series *Hurwitt* (8) included 12 patients with various chronic generalized skin disorders peripheral lymph node enlargement and eosinophilia in the peripheral blood Histologically the lymph nodes showed lipomelanotic reticulosis These patients were followed over varying periods maximum 4 years None of the patients developed malignancy In some cases complete remission of the lymph node enlargement was reported

Soloff (27) described a 17 year old male with pulmonary miliary tuberculosis concurrently with inguinal lymph node enlargement Biopsy of a lymph node showed lipomelanotic changes Several years previously the patient had had a squamous and extremely itching skin affection in the inguinal regions

In their study *Poul Larkin et al* (22) included 6 children between 2 months and 3 years of age The children had chronic generalized eczema which was refractory to almost any kind of therapy There was also a pronounced generalized enlargement of the lymph nodes and eosinophilia in the peripheral blood Lymph node biopsy revealed typical lipomelanotic changes in all the children The ages of the patients deserve special notice since most described cases refer to adults in particular about the age of 50 years 3 times as often in males as in females (*Lennert*)

In all the above papers the benign nature of the changes was emphasized But in 1939 *Pautrier & Woringer* (25) published a report concerning a 53 year old male with a skin disease which started as an itching erythema of the right leg and became generalized in the course of a few weeks One year later a generalized peripheral lymph node enlargement had developed The histological picture of a lymph node showed at this time a typical lipomelanotic reticulosis Repeated lymph node biopsy 11 months later revealed a completely changed

structure The capsule of the lymph node was thickened Periadenitis was present In the lymph node itself no normal lymph follicles, no reticulum cell proliferation and no deposits of fat or melanin were seen, but numerous cells were observed similar to those found in the skin in mycosis fungoides Histological examination of a biopsy from an ulcerating skin tumour from the patient revealed typical mycosis fungoides

In 1950 the above report was supplemented by 2 cases of lipomelanotic reticulosis reported by *Bluefarb & Webster* (3) Also these cases were supposed to have been transformed into mycosis fungoides Finally, *Oliver & Greenberg* (21) described a patient with erythrodermia and lymph node lesions indicative of lipomelanotic reticulosis The patient died a few months later from mycosis fungoides

In his material, *Laipply* (13) included 6 patients with itching dermatitis and moderate lymph node enlargement Microscopic examination of extirpated lymph nodes from all the patients revealed lipomelanotic reticulosis Besides, 2 patients had monocytic leucaemia The author concluded that the lipomelanotic changes were of a benign nature, secondary to the chronic pruriginous dermatitis, and that there was no relationship to leukaemia, malignant lymphogranulomatosis or lymphosarcoma, although the above changes might appear concurrently with diseases

Lennert (15, 16) pointed out that lipomelanotic reticulosis carries a good prognosis, whereas the skin disease per se may prove fatal after a relatively brief duration of illness The author also questioned the above-mentioned transition to malignant disease

It is thus a matter of dispute whether lipomelanotic reticulosis should be considered a purely benign condition or whether it may be transferred into a malignant disease as *e g* mycosis fungoides, malignant lymphogranulomatosis or lymphosarcoma This difference of opinion is justified, since the small number of patients observed with lipomelanotic reticulosis associated with malignant diseases occurring simultaneously or subsequently, is too limited to allow of a definite conclusion Furthermore it should be pointed out that although 2 biopsy specimens from the same patient, obtained at a certain interval, first have revealed changes as those seen in lipomelanotic reticulosis and later on changes as those seen in malignant disease, this should not necessarily be interpreted as being a transition to malignant disease Nevertheless, the cases observed will give impetus to careful observation of patients with lipomelanotic reticulosis with a view to the subsequent fate of such patients

As regards the difficulties in connection with the differential diagnosis which may arise in lymph node enlargement in particular, the various materials show good agreement The clinical diagnosis will often be one of the malignant diseases mentioned above, until the result of microscopic examination of a lymph node biopsy is available *Combes*

& *Bluefarb* (5) described 15 patients with various cutaneous manifestations and superficial lymph node enlargement 9 out of the 15 patients had eosinophilia The clinical picture pointed towards Hodgkin's disease In all of the 15 patients lymph node biopsy was performed Microscopic examination revealed changes corresponding to giant follicular lymphadenopathy Subsequently 5 of the patients developed polymorph cell sarcoma *Agress & Fishman* (1) mentioned a 68-year old male with brownish pigmentation of the skin and peripheral lymph node enlargement The clinical diagnosis was generalized melanocarcinoma However, microscopic examination of a lymph node showed lipomelanotic reticulosis Another patient had an eczematous, pruriginous skin disease accompanied by lymph node enlargement The clinical diagnosis was Hodgkin's disease or mycosis fungoides Also in this case the microscopic examination of a lymph node revealed lipomelanotic changes

On the other hand the histological examination of a lymph node may give rise to an incorrect diagnosis Thus, *Moller* (18) in the course of his study in the pathological institute in Lund, Sweden, carried out a histological revision of 300 lymph nodes which were examined in the institute over a 6 year period During this revision he found 9 cases with lipomelanotic changes The previous histological diagnosis was Hodgkin's disease in 3 cases, of which 2 were considered to be certain, while the third case was more doubtful In the remaining 6 cases the diagnosis was non specific lymphadenitis In most cases the clinical diagnosis were pruriginous eczema or erythrodermia, in a couple of cases also Hodgkin's disease All the patients had superficial lymph node enlargement

Consequently a careful histological examination of the lymph nodes is of decisive importance for a definite diagnosis of lipomelanotic reticulosis However, also bone marrow examinations may reveal cytological changes resembling those previously described in lymph nodes Such changes will be discussed for the first time in the present paper

At the Radium Centre of Jutland we have observed a patient, in whom we consider the diagnosis of lipomelanotic reticulosis to be highly probable on account of characteristic cellular findings in the bone marrow

CASE REPORT

The patient is a 12 year old boy who was first admitted in February 1963 to the Radium Centre in Århus for examination with a view to a possible malignant lymphogranulomatosis Hodgkin (Record No 3091 62 63)

In the beginning of December 1962 the patient was admitted to the local hospital with fever and in a poor general condition and furthermore with pain in the right shoulder and the right side of the chest at deep resp[illegible] diagnosis of right [illegible] with penicillin and [illegible] of these drugs had [illegible]ating At the end of [illegible] according to the method of

Daniel Microscopic examination revealed a fairly well preserved structure of the lymph node tissue There were some lymph follicles with fairly large secondary centres In between the follicles moderate reticulum cell proliferation was observed A number of eosinophilic granulocytes was scattered through the tissue By contrast no definitely pathological reticulum cells were seen no Sternberg s giant cells no fibroses The pathologist concluded that most likely it was moderate non specific reactive changes but that it might be Hodgkin s disease in an early phase

The patient was then treated with phenylbutazone his general condition improved and the temperature became normal Since the diagnosis was uncertain the patient was transferred to the Radium Centre in Arhus

Previous history The family history was negative as regards hereditary diseases blood diseases malignant tumours or allergic diseases in particular no cutaneous manifestations The patient has two brothers who are healthy Also his parents are healthy Since the age of 6 months the patient has showed a tendency to formation of small recurrent abscesses in various regions The last 3 years there have been rather sporadic but troublesome skin changes of an eczema like nature and localized to the eye lids the vestibulum nasi and the axillary regions The skin affection has been moderately pruriginous especially in the axillae and the patient has therefore scratched these regions very much The patient is said to be unable to tolerate penicillin but there is no information about the manifestations of this hypersensitivity Apart from this the patient has never shown any signs of allergy and he has never had symptoms of asthma or hay fever

On the 1st admission to this hospital the general condition of the patient was found to be good he was a little pale but had no dyspnoea cyanosis or jaundice Temperature 36 8° C. Blood pressure 115/50 mm Hg On either side of the neck there were small indolent freely movable lymph nodes In the axillae there were a few slightly enlarged lymph nodes Otherwise the physical examination revealed no

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per mm³

Differential count revealed 10–14 per cent eosinophilic granulocytes otherwise normal distribution A sternal marr w puncture showed slightly hyperplastic bone marrow with moderate eosinophilia but no formation of giant cells The sero reac tions for ornithosis toxoplasmosis and the Rose Waaler reaction were negative and no L E factors were demonstrated Cold haemagglutinin titre was normal The chest roentgenogram *revealed pleural changes basally on the right side* with rounding of the right sinus phrenicocostalis but on comparison with roentgenograms taken at the local hospital the changes were regressing The patient was discharged without

ell during the first 6 months The following
the Radium Centre took place in Novemb er
tite and occasionally he ran a temperature
ye lid and in the vestibulum nasi was very
y a dermatologist with unguentum hydr
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ely in the course of one week s *treatment*

with either drug but the affection re urred as soon as treatment was discontinued

On the 2nd admission an eczemat us affection of the right upper and lower eye lids was found together with a scabby affection in the vestibulum nasi In the right axilla a smallspotted scratched skin region was observed There was a very slight brownish pigmentation of the skin On either side of the neck in the left axilla and in the inguinal regions there were palpable slightly enlarged indolent lymph nodes There was no palpable enlargement of liver or spleen Remaining ex aminations Haemoglobin 11 2 12 8 g per 100 ml ESR 63 5 mm hour Leucocyte count 5200 9000 per mm³ Repeated differential counts revealed eosinophilia maximum 14 per cent Haematocrit 35 41 per cent Normal platelet counts Serum Cu 207 166 μg per 100 ml Paper electrophoretic analysis of rum was normal Total protein in serum 7 8 g per 100 ml No urinary excretion of melanin Ophthalmological examination revealed moderate eczema of the ye lids otherwise normal conditions During the first days after admission the temperature was raised



Fig 1

Smear preparation of the sternal marrow Two reticulum cells containing abundant pigment are seen May Grunwald Giemsa × 400

maximum 38 6° C. Chest roentgenogram showed further regression of the former pleural changes on the right side and no fresh infiltrations were seen Results of blood culture were negative During the last 3 weeks before discharge the temperature remained normal

Lymph node biopsy from the right side of the neck (December 4th, 1963) The lymph node was well defined and had a normal structure with lymphoid tissue and a few slightly enlarged germinal centres There were no specific inflammatory changes no signs of systemic disorders or malignancy Microscopic diagnosis Lymph node with simple hyperplasia (*Steen Olsen*) Sternal marrow puncture (December 4th 1963) showed pronounced hyperplasia slight displacement to the left of the granulopoiesis and pronounced eosinophilia The erythropoiesis was normal and purely normoblastic The thrombopoiesis appeared slightly hyperplastic a great many platelet forming megacaryocytes being present Furthermore, there was a definite increase in the number of plasma cells and reticulum cells Several of the cells seemed to be in the course of transition from plasma cells to reticulum cells They contained a fairly small roundish very loosely structured nucleus which was situated very excentrically and with abundant chromatin content In the abundant cytoplasm numerous fine to fairly coarse bluish inclusions fairly large vacuoles and in a few cells furthermore brownish granules were seen (Fig 1) After special staining with potassium ferricyanide bluish granules were seen in some of the reticulum cells indicating the presence of ferruginous pigment Furthermore a pronounced content of fat was demonstrated in some of the reticulum cells after staining with Sudan black Finally examination for melanin was performed by means of the Masson Fontana staining method which is based upon the ability of the melanin to reduce solutions of ammoniacal silver nitrate to metallic silver The result was distinctly positive (*J Bichel*)

These cells were of the same type as those described in lymph nodes in lipomelanotic reticulosis They must be reticulum cells, which contain fat and partly iron containing, partly melanin-containing pigment

By repeated studies of the previous smear preparations of sternal marrow from February 1963 the characteristic cells described above were found

Skin biopsy (December 10th 1963) from the anterior surface of the right femur from a region with increased pigmentation showed a markedly pigmented normal section of skin without signs of systemic diseases inflammation or malignancy (*Steen Olsen*)

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nisone treatment was completely discontinued in the course of 3 months The treat ment had a good effect on the skin symptoms, which disappeared completely When last seen in the out patients department in June 1964 the patient remained well In the left axilla a couple of slightly enlarged lymph nodes were palpable but in other regions no lymph nods enlargement was found The spleen was not palpable The appearance of the skin was everywhere normal

DISCUSSION

Only relatively few descriptions of bone marrow examinations in patients with lipomelanotic changes of the lymph nodes are available In the literature reviewed the author found 14 patients, in whom the sternal marrow was examined simultaneously with the appearance of skin manifestations, peripheral lymph node enlargement and the changes of the lymph nodes which are characteristic of lipomelanotic reticulosis In a few patients the sternal marrow was reported to be completely normal (*Neuhold & Wolfram* 1952) However, moderate changes were seen in most cases The most frequent finding was an increased number of eosinophilic granulocytes (*Sulzberger* 1939, *Laipply* 1948, *Bluefarb & Webster* 1950, *Hall-Smith* 1950 and *Jarrett & Kellett* 1951) Less frequent findings were displacement to the left of the granulopoiesis (*Rothman, Scherber & Laden* 1949, *Neuhold & Wolfram* 1952 and *Loblich & Wagner* 1953) and an increased erythro poiesis (*Agress & Fishman* 1950 and *Laipply* 1951) In one of their patients, *Neuhold & Wolfram* described more pronounced bone marrow changes, which may suggest lipomelanotic changes The patient was a 55-year-old male who had for 12 years suffered from a pruriginous, eczematous skin affection and peripheral lymph node enlargement There was eosinophilia (27 per cent) in the peripheral blood Microscopic examination of a lymph node revealed pronounced proliferation of reticulum cells and accumulation of large cells with a melanin-containing pigment Examination of the sternal marrow showed an increased number of eosinophilic granulocytes Furthermore, diffuse proliferation of reticulum cells and plasma cells was seen together with scattered islets of reticulum cells and large lymphoid plasma cells Some of the plasma cells were atypical giant-cell like and with large vacuoles Any observation of pigment in these cells was not reported

In the patient described in the present paper, the pronounced bone marrow changes as *e g* the reticulum cell proliferation, the increase in the number of eosinophilic granulocytes and plasma cells and the high content of fat and pigment in some of these cells, were exactly the features which suggested the diagnosis of lipomelanotic reticulosis At the same time a lymph node biopsy from the right side of the neck was performed The result of the histological examination was the finding of a simple hyperplasia of the lymph node One year previously a lymph node biopsy according to the method of Daniel had been carried out At that time a sporadic, very slight reticulum cell proliferation was described, together with very few scattered eosinophilic

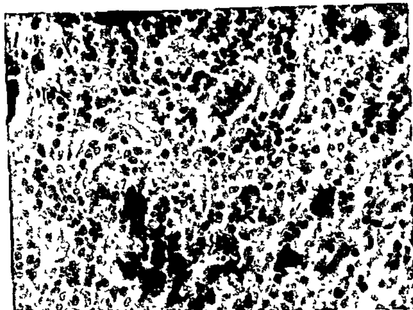


Fig 2
The dominant changes in the lymph node are the deposited pigments phagocytosed by the proliferating reticulum cells Masson Fontana × 400

granulocytes and a few plasma cells The pathologist ventilated that it might be malignant lymphogranulomatosis at an early stage However, neither the examination results nor the course of disease up till the present could confirm this diagnosis The finding of melanin-containing cells in the bone marrow occasioned the performance of investigations with a view to malignant melanoma or metastases from a melanoma No parts of the skin were suspicious of melanoma There was no urinary excretion of melanin Finally the eye examination showed normal conditions, apart from a moderate eczema on the eyelids

The lymph node examination carried out in December 1962 was reviewed In a specimen stained with haematoxylin eosin a moderate proliferation of the reticulum cells was seen, some of which contained [illegible] tinctly stained content of pigment were seen (Fig 2) No reaction of these cells was obtained after iron staining The diagnosis of lipomelanotic reticulosis was confirmed by these changes in connection with the patient's pruriginous, eczematous skin disease, the moderate lymph node enlargement, the eosinophilia in the peripheral blood and the bone marrow changes

Several authors (*Pautrier & Woringer* 1932, *Laipply* 1948, *Bluefarb*

& *Webster* 1950) mention that the increased content of pigment in the lymph node is presumably due to the fact that the itching skin affection produces scratching by which melanin is formed and released from the epidermic cells The melanin is carried via the lymph vessels to the regionary lymph nodes, where it is phagocyted by the reticulum cells which proliferate The increased fat content might be explained by the same mechanism The theory is supported by the fact that by far the greater majority of the patients with lipomelanotic changes in the lymph nodes have indeed a pruriginous skin affection Further more, one of De Paula and Hohnadll's series of animal experiments must be borne in mind This paper was cited by *Lennert* (15) By injecting melanin the authors succeeded in producing changes in the experimental animals analogous to those observed in man It tells against this theory that in patients with a quite similar pruriginous *skin affection reticulum cell proliferation may occur in the lymph* nodes, but *without* phagocytosis of melanin and fat Likewise, only few patients develop lipomelanotic changes when they are exposed to a large supply of melanin (*Lennert*)

In this connection a report from *Verloop et al* (29) may be of interest They describe 2 sisters who—in addition to their being albinos—showed prolonged bleeding time and anomalous pigment cells in the bone marrow The syndrome was first described in 1959 by *Hermansky & Pudlak* In both patients smears of bone marrow showed large pathological cells with a small excentric nucleus and dense, bluish pigment granules in the cytoplasm The thrombopoiesis, the leucopoiesis and the erythropoiesis were not changed markedly Various cytochemical special examinations were carried out in order to characterize the observed *pigment* more particularly It seemed to be a ceroid pigment (chromolipoid) The authors were not able to find any correlation between the disturbed formation of melanin in the presence of the albinism and the pigment cells observed, but discussed the possibility whether the pigment might originate from reduction of haemoglobin because of bone marrow haemorrhage

Finally it should be mentioned that in our department we have observed a few large cells with coarse bluish pigment granules in the cytoplasm in smears of sternal marrow from patients with malignant lymphogranulomatosis Hodgkin and from one patient with myeloid leucosis Attempts at a more detailed identification of this pigment have not been made

Hence, abnormal pigment *cells in the bone marrow may be seen* in various diseases Although the pigment granules in the previously mentioned three groups of patients have had a certain morphological and tinctorial resemblance in May Grunwald-Giemsa stained specimens, the cytochemical special investigations have revealed a definite difference in the two first mentioned groups of diseases, in which the pigments are melanin and ceroid, respectively

The changes in bone marrow and lymph nodes demonstrated in the patient described in the present paper may indicate an involvement of the entire reticulo endothelial system. It remains to be clarified why then some patients apparently exhibit changes in well defined regions only whereas in other patients, perhaps the minority, the entire reticulo endothelial system is involved. It may well be that a special hereditary tendency is responsible which tendency may influence the subsequent fate of the lipomelanotic changes and their possible transformation into a malignant disease.

SUMMARY

Lipomelanotic reticulosis is briefly described and a case of this disorder in a 12 year old boy is reported. The patient has for 3 years had a pruriginous eczematous skin affection of the eye lids, in the vestibulum nasi and the axillae associated with a very slight lymph node enlargement on the neck and in the axillary and inguinal regions. Examination of the sternal marrow reveals changes which completely resemble the changes described in lymph nodes in patients with chronic itching skin disorders and superficial lymph node enlargement. The dominant features are reticulum cell proliferation, deposits of fat and melanin and eosinophilia. When reviewing the findings at microscopic examination of a lymph node removed previously, the changes which are characteristic for lipomelanotic reticulosis are found.

In the literature reviewed sternal marrow examinations were reported in 14 patients with lipomelanotic reticulosis but in none of these patients identical characteristic changes in bone marrow and in lymph nodes are described, in particular no abnormal pigment cells.

As far as the author knows this is the first time that a lipomelanotic reticulosis is diagnosed on the basis of a bone marrow examination.

The patient concerned was treated with prednisone for a brief period. Under this treatment the skin affections disappeared. The patient has been followed for approx. 2 years and during this period no progression of his disease has been observed.

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LABORATORY INVESTIGATION OF ARCTIC STRAINS OF RABIES VIRUS

By

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Received 31 x 64

During the spring of 1961 and subsequently this laboratory received a number of dog and fox heads from Thule AFB, Greenland, for rabies examination A high percentage (40 4) of these specimens contained rabies virus as detected by the fluorescent rabies antibody (FRA) and mouse inoculation tests Microscopic examination of Sellers stained impression smears were all negative for negri bodies Attempts to subpass this agent in mice gave either negative or inconsistent results, thus making virus identification by the serum virus neutralization test impossible

Although the existence of rabies had been recently confirmed in foxes and dogs in Greenland (*Jenkins & Wamberg* 1960), our failures to subpass the virus in mice and to demonstrate negri bodies in brain smears presented a diagnostic problem A study was therefore initiated to confirm the condition in these animals as rabies and to investigate some of the biological properties of the virus The present paper is a report of our studies with the arctic strains of rabies virus

MATERIALS AND METHODS

Virus Isolations All isolations were made from 10 per cent brain or salivary gland tissue suspension prepared in 10 per cent rabbit serum saline Subsequent virus pools were prepared as 20 per cent tissue suspensions and stored at —60° C Later in the course of study the diluent was changed to 10 per cent [illegible] from 6–7 day emb [illegible]

[illegible] of the disease or by the standard neutralization test for identification of the virus

Virus Titrations All viruses were titrated by the intracerebral inoculation of tenfold dilutions of virus into each of five mice or hamsters The titer was cal

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1 Robert A [illegible] is a Major in the United States Air Force Veterinary Service

culated by the method of *Reed & Muench* (1938) Titers are expressed in mouse lethal dose 50 per cent (MLD_{50}) and hamster lethal dose 50 per cent (HLD_{50})

Serum virus Neutralization Tests The technic employed was as described in the WHO monograph Laboratory Techniques in Rabies (1954)

Viruses Heads of arctic foxes and husky dogs were received frozen from Thule AFB Greenland The fixed strain of rabies virus (CVS 25) was obtained from the National Institutes of Health

Laboratory Animals 3 to 4 week old white Swiss mice (Fairchild strain) 4-5 week old Syrian hamsters 250-300 gram guinea pigs and (3 4 pounds) New Zealand rabbits were utilized

Routes and Amounts of Inoculum *Mice* were inoculated with 0 03 ml intracerebrally *hamsters* with 0 05 ml intracerebrally intramuscularly and intraperitoneally and with 0 1 ml subcutaneously *guinea pigs* with 0 1 ml intracerebrally and intramuscularly and with 1 0 ml intraperitoneally and *rabbits* with 0 25 ml intracerebrally 0 75 ml intramuscularly and 1 0 ml subcutaneously

Collection of Saliva Samples Saliva samples were collected by swabbing the mouths of the inoculated animals with cotton tipped applicators soaked in 1 0 ml of 10 per cent rabbit serum saline containing 1000 units of penicillin and 2 0 mg streptomycin Prior to animal inoculation the mixture was incubated at 4° C for 1 hour

Preparation for Histologic Examination Direct brain smears were stained by *Sellers* (1927) method Representative brain tissue was fixed in Zenker s acetic fluid Sections were stained by the *Schleifstein* (1937) technique

Fluorescent Antibody (FRA) The brain impression smears were fixed in acetone at —20° C for four hours The conjugated antiserum was obtained from the Reagent Section Communicable Disease Center Atlanta Ga The staining procedure was that described by *Goldwasser & Kissling* (1958) All preparations were examined under ultraviolet light with an Osram HBO 200 watt light source and a Leitz Ortholux VAM microscope

Ether Susceptibility A 20 per cent brain suspension of virus was added to anesthetic ether to a final concentration of 20 per cent ether mixed and held at 4° C for 18 hours The control sample was handled in the same manner but without ether Following evaporation of the ether the virus was titrated in mice

RESULTS

Isolation of the Arctic Rabies Virus The earliest signs of illness in mice were a ruffling of the hair and humping of the back Although illness was observed as early as the 8th postinoculation day most of the mice were asymptomatic until the 11th or 12th day Death generally followed within two days, but it was not uncommon for some mice to exhibit symptoms for 7 days before death Some animals would exhibit humping between the 12th and 15th day and become normal in appearance and thereafter remain normal throughout a 30 day observation period Mice succumbing from infection became paralyzed and usually died in a humped position Ill mice did not become irritable or hypersensitive Numerous Sellers stained impression smears from the dead mice were examined for negri bodies Although a few bodies were observed in some smears they were not considered typical negri bodies and rabies was not diagnosed on the basis of these smears The fluorescent antibody technic was applied to smears from brains of mice dying and in every instance a positive immuno fluorescence was observed Of the 47 specimens reported the same 19 (11 foxes and 8 dogs) were positive for rabies by both the mouse inoculation test and the fluorescent antibody technic All Sellers stained impression smears

of the original animals were negative for negri bodies. Negri bodies were demonstrated in sections of brain from different species of laboratory animals at various animal passages in the 10 isolates tested.

The relationship between the virus concentration in some of the fox brains and the time of death of mice was determined. These results are shown in Table 1. The time of death in the animals inoculated with the brain having the lowest virus concentration (1.5 logs) was 13 days, whereas those inoculated with the highest concentration of virus (3.333 logs) died in an average of 14.3 days. The shortest average time of death was observed in the mice receiving an inoculum containing 2.833 logs of virus.

TABLE 1

Amount (MLD_{50}/0.03 ml) of Rabies Virus in 10 Per cent Suspension of Original Animal Brains

Animal number	Titer (log LD_{50})	Mean day of death: Isolation attempt 1	Mean day of death: Isolation attempt 2	Mean day of death
F 199	3.333	15.6	13.0	14.3
F 200	2.375	16.3	15.8	16.0
F 206	2.833	10.0	9.0	9.5
F 207	1.500	13.0		13.0
F 209	2.625	16.4	18.2	17.5

Two of the arctic strains (209 and 219) were tested in mice and hamsters to determine the suitability of these two hosts for primary isolation of the virus.

Ten percent brain suspensions from these 2 foxes were inoculated intracerebrally into mice and hamsters. The brains from dead or prostrate animals were pooled and two additional passages made in the respective species. The third animal brain passage material was titrated intracerebrally in both mice and hamsters.

The results of this comparative study are summarized in Table 2. The average number of days from the time of inoculation until the time of death of hamsters for the primary isolation of the virus was approximately 5 days shorter than in the mice. In the third animal passage the average time of death in hamsters decreased to less than one half of that observed in mice. In all hamster passages the mortality rate was 100 per cent, whereas in the mice the percent mortality varied between 71.4 per cent and 100 per cent. In every instance the mortality rate was less in mice than in hamsters on primary isolation. With one exception the viruses titered higher in hamsters than in mice. However this one strain had been isolated and [illegible] passed [illegible] mouse [illegible] 100 or

TABLE 2

Comparison of Susceptibility of Mice and Hamsters for Primary Isolation of two Arctic Strains of Rabies Virus

is n ox n	Passage number	Mean day death		Percent mortality		Titer (log LD50)			
						Virus from mouse passage		Virus from hamster passage	
		Mice	Hamster	Mice	Hamster	Mice	Hamster	Mice	Hamster
9	Primary Isolation	18 2	13 5	71 4	100 0				
	1	14 2	9 0	83 3	100 0				
	2	16 2	8 0	83 3	100 0	2 28	3 0	4 37	4 16
9	Primary Isolation	17 3	11 0	80 0	100 0				
	1	8 3	9 0	100 0	100 0				
	2	14 3	6 0	100 0	100 0	1 5	2 83	5 0	5 75

more, the hamster-adapted virus was used in mice for identification purposes

The hamsters reacted to infection quite differently than did the mice Illness and death were both observed 4 to 8 days sooner in hamsters Generally, the hamsters showed signs of illness for only a day or two before death They were hyperactive, irritable, and at times very aggressive Death was rapid and cannibalism was high among cagemates

TABLE 3

Transmission Studies in Rabbits with 3rd Hamster Brain Passage of Arctic Rabies Virus (219)

Animal number	Age	Route	Dose HI D50	Day of termination		Virus recovery	Antibody response
				Death	Killed		
24	Adult	SC	1 000 000		19*	—	+
15A	Adult	SC	1,000 000		46	—	—
5	Adult	SC	1 000 000		46	—	—
VO	Adult	IM	750 000		46	—	—
0	Weanling	IC	12 000	17		—	—
2	Weanling	SC	12 000		90	—	—
3	Weanling	SC	12 000	19		—	NT§
4	Weanling	IM	12 000	79		—	NT§
5	Weanling	IM	12 000		90	—	+
6	Weanling	IM	12 000		90*	—	+

* Killed with Symptoms

§ Not Tested

Results of Rabbit Transmission The responses of adult and young rabbits to different dose levels of the virus are given in Table 3 The

animals inoculated by the subcutaneous and intramuscular routes developed symptoms between the 12th and 16th days The first clinical sign of illness was a *slight incoordination* of the extremity in which the inoculum was administered, with subsequent paralysis Of these animals the rabbits inoculated by the intramuscular route survived longer than those inoculated subcutaneously One paralyzed rabbit was observed for 90 days before sacrificing The rabbit inoculated intracerebrally was salivating profusely on the 15th postinoculation day, and developed CNS disorders a day later Signs consisted of hypersensitivity, biting motions at its flanks, and posterior paralysis, followed by death on the 17th day

Three of the 8 rabbits tested for neutralizing antibodies were positive Although the brain smears from the rabbits inoculated intracerebrally and subcutaneously were positive for rabies antigen by the fluorescent antibody technique, the virus was not isolated in mice Hamsters and weanling rabbits inoculated intracerebrally with brain suspensions from these two rabbits were also negative for rabies Intracellular bodies similar to negri bodies were observed in stained sections from these 2 rabbits, however, they could not be definitely identified as negri bodies

Tissue neutralization tests were performed on the brain suspensions from rabbits number 0 and 3 (see Table 3) according to the method described by *Carski, Wilsnack & Sikes* (1962) Their respective titers of virus inhibition were 1 291 and 1 9

TABLE 4
Transmission Studies in Hamsters with 12 000 HLD_{50} Doses of Arctic Strain Rabies Virus (219)

Number of animals inoc	Route of inoc	Deaths		Mean day of illness	Mean day of death	Virus recovery
		No dead / No inoc	% dead			
5	IC	5/5	100	6	7	+
5	IM	3/5	60	10 6	13	+
5	IP	5/5	100	4 6	4 8	+

Hamster Transmission Studies Results of transmission in hamsters infected with 12,000 HLD_{50} doses of 219 are presented in Table 4 Thirteen of the 15 hamsters inoculated (5 I C , 5 I M , and 5 I P) died with symptoms of the disease The shortest mean day of death occurred in animals inoculated by the intraperitoneal route (4 8), with the longest (13) being in those animals inoculated intramuscularly The two animals surviving the intramuscular inoculation were observed for a period of 30 days Virus was recovered from 7 of 8 brains tested Brains from three hamsters inoculated by the intraperitoneal route were tested and 2 of these were positive for virus

Guinea Pig Transmission Studies Twenty-two guinea pigs were inoculated with 1200 HLD_{50} doses of strain 219 virus by various routes The results are summarized in Table 5 The eight animals inoculated intracerebrally died with a mean day of death of 11 3 Five of the 9 or 55 5 per cent of the animals inoculated intramuscularly died with a mean day of 15 All five animals receiving an intraperitoneal injection survived a 60 day observation period Virus was recovered from brains of the dead animals which were inoculated by the intracerebral and intramuscular routes A slight antibody response was demonstrated in those animals which survived Eight additional guinea pigs inoculated subcutaneously with 12000 HLD_{50} all died between the 18th and 28th days

TABLE 5

Transmission Studies in Guinea Pigs with 12 000 HLD_{50} Doses of Arctic Strain Rabies Virus (219)

Number of animals inoc	Route inoc	Deaths		Mean day of illness	Mean day of death	Virus recovery	Antibody response
		No dead / No inoc	% dead				
8	IC	8/8	100 0	9 5	11 3	+	NT*
9	IM	5/9	55 5	13 4	15 0	+	+
5	IP	0/5	0 0			NT	+

* Not Tested

Serologic Studies All isolates studied were identified as being rabies virus by serum neutralization tests utilizing the constant serum-variable virus concentration system Immune serum prepared against fixed (CVS) virus was used in all neutralization test for the identification of the isolants The identification of strain 209 was confirmed by Dr *R K Sikes*, Rabies Investigation Laboratory, Communicable Disease Center

Ether Studies Exposure of the two arctic viruses to 20 per cent ether for 16 hours at 4° C resulted in a 95 3 to 97 8 percent loss in infectivity for mice

Saliva Studies All attempts to isolate the virus from the saliva of infected rabbits and guinea pigs were unsuccessful In several instances the inoculation of saliva resulted in deaths but rabies virus was not identified as the causative agent by immunofluorescence nor were the agent reisolated

Morphological Studies Histological studies were performed on brain tissue from mice, guinea pigs, hamsters, and rabbits infected with the different isolants of arctic virus

Sections of brains from these animals showed a mild to moderate meningoencephalitis characterized by lymphocytic infiltration, perivascular cuffing, and some gliosis With the exception of the two rabbit brains examined, inclusion bodies were demonstrated in all animal

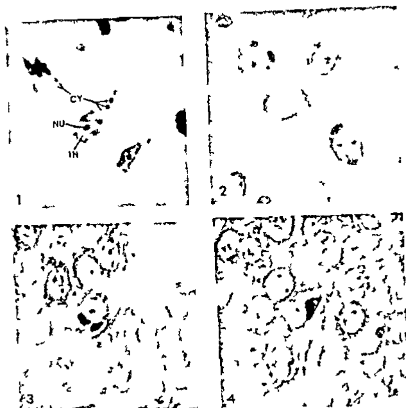


Fig 1 Cytoplasmic and intranuclear negri bodies in nerve cell from hamster brain inoculated with arctic rabies virus CY cytoplasmic NU nucleolus IN intranuclear Schleifstein stain × 940
Fig 2 Cytoplasmic negri bodies in nerve cells from mouse brain inoculated with × 730 AFIP 62 5769
Fig om mouse brain inoculated with) AFIP 62 5767
Fig om mouse brain inoculated with AFIP-62 5768

species inoculated with 10 different isolates from foxes However they were not all observed at the time of the primary isolation

The inclusion bodies appeared in various forms and were demonstrated in the neurons of the cerebral cortex hippocampus and cerebellum They occurred most frequently as small (0 5 3 0 μ) eosinophilic spherical cytoplasmic bodies with little or no internal structure (Fig 1) In some preparations larger irregular or oval shaped bodies containing one or two large basophilic granules were observed (Figs 2 4) These larger inclusions were 3 5 μ and the granules measured 1 2 μ in diameter Intranuclear inclusions in nerve cells were demonstrated in some sections (Fig 1) Their occurrence and numbers varied among the different strains of viruses studied They appeared as round small (0 5 1 5 μ) eosinophilic bodies with a well defined border The

intranuclear inclusions occurred as single structureless bodies within the nuclei, whereas the number of cytoplasmic inclusions varied from one or more per cell

DISCUSSION

In reference to the difficulty in establishing some of the Arctic strains in mice several possible factors were considered The presence of latent infections in the mice was ruled out following unsuccessful attempts to demonstrate the existence of mouse pathogens However, organisms compatible to *Encephalitozoon cuniculi* were observed in the brain of one mouse inoculated with a brain suspension from an Arctic fox It was never determined whether the organisms were in the mouse or came from the inoculum The mice from the same source are being utilized in this laboratory routinely for other virologic studies without apparent problems The postulate of "self-sterilizing neuro-infections" or interference phenomenon was considered Adaptation to mice was not enhanced when brain suspensions were diluted beyond 10 per cent concentration The demonstration of an inhibitory substance in the two rabbit brains suggests that a local tissue neutralizing factor is responsible for the non-infectiousness of the virus Similar observations have recently been experienced in wild-life rabies studies (*Sikes* 1962) Salivary glands from skunks, which were shown to contain specific fluorescence for rabies, failed to infect mice when inoculated intracerebrally Tissue from these glands when treated as a serum in a serum-virus neutralization test inhibited fixed virus

The apparent recovery of clinically sick mice following inoculation observed in this laboratory was also reported by *Jenkins & Wamberg* (1960) Although clinical signs of illness in mice are not diagnostic for the presence of rabies virus, in some groups of mice cagemates would succumb and others would recover In those who died rabies antigen could be demonstrated by the FRA technic Various attempts to re-isolate the virus and reduce the incubation period in mice by blind passing brains at different intervals following inoculation failed The experimental data indicate no significant correlation between the amount of virus in the brain and the average time of death in the mice

Although the virus was not isolated from the saliva of the laboratory animals it did not possess other characteristics associated with fixation Infections were produced by parenteral inoculations with demonstrable inclusion bodies in the brain tissues With the experience of not isolating the virus from infected rabbits and some mouse brains which were fluorescent positive for rabies it is possible the virus is in saliva and salivary glands but does not manifest itself by producing illness upon passage

The demonstration of negri bodies with marked morphological variations in laboratory infected animals offers an explanation for the failures in the recognition of negri bodies in stained brain smears of the

arctic animals. Although similar bodies were observed in brain smears of mice they were not considered diagnostic for negri bodies. Of the four positive cases of rabies in dogs and foxes as reported by *Jenkins & Wamberg* (1960) negri bodies were demonstrated in the brains of each species. These authors did however observe atypical negri bodies on a Sellers stained brain smear from a paralyzed mouse sacrificed on the 15th post inoculation day. In an earlier report *Kantorovich* (1957) reported that a virus appearing to be a variant of rabies was the cause of polar madness in polar animals. He reported that inclusions were more rarely found in these animals than in typical rabies. More recently *Kantorovich et al.* (1963) reported that inclusions could not be demonstrated in brains of polar foxes experimentally infected with strains of rage (rabies) virus isolated from polar foxes in the far north of the USSR. However, when the same material was inoculated into white mice and rabbits cytoplasmic inclusions were found regularly. They concluded that the absence of inclusions in polar foxes is a distinctive sign of the investigation form of rabic infection. Since the dog and fox heads were received frozen the possibility of the negri bodies becoming distorted due to the freezing and thawing of the brain tissue was considered.

The presence of intranuclear inclusion bodies with these arctic strains is of particular interest since negri bodies associated with rabies infection are generally found in the cytoplasm of the nerve cells. However, *Kantorovich* (1957) described similar acidophilic inclusions in the nuclei of nerve cells in animals infected with the virus of polar madness (rabies). *Sokolov & Vanog* (1962) reported on the nature of intranuclear inclusions in experimental rabies employing FRA, acridine orange and aniline dye staining methods. Although these authors demonstrated the greatest concentration of rabies virus in the cytoplasm, intranuclear inclusions did occasionally contain rabies antigen. Positive intranuclear immunofluorescence specific for rabies virus has been demonstrated in this laboratory but extensive studies to correlate theses inclusions specifically with immunofluorescence have not been completed. Additional work is in progress to further characterize these intranuclear bodies.

The possibility of the arctic strain being a variant or aberrant form of rabies necessitates additional studies.

SUMMARY

Viruses identified as rabies by the demonstrat[illegible]

[illegible] methods for detection of the virus, the fluorescent antibody technic and mouse inoculation method were shown to be equally sensitive.

intranuclear inclusions occurred as single structureless bodies within the nuclei, whereas the number of cytoplasmic inclusions varied from one or more per cell

DISCUSSION

In reference to the difficulty in establishing some of the Arctic strains in mice several possible factors were considered The presence of latent infections in the mice was ruled out following unsuccessful attempts to demonstrate the existence of mouse pathogens However, organisms compatible to *Encephalitozoon cuniculi* were observed in the brain of one mouse inoculated with a brain suspension from an Arctic fox It was never determined whether the organisms were in the mouse or came from the inoculum The mice from the same source are being utilized in this laboratory routinely for other virologic studies without apparent problems The postulate of "self-sterilizing neuro infections" or interference phenomenon was considered Adaptation to mice was not enhanced when brain suspensions were diluted beyond 10 per cent concentration The demonstration of an inhibitory substance in the two rabbit brains suggests that a local tissue neutralizing factor is responsible for the non-infectiousness of the virus Similar observations have recently been experienced in wild-life rabies studies (*Sikes* 1962) Salivary glands from skunks, which were shown to contain specific fluorescence for rabies, failed to infect mice when inoculated intracerebrally Tissue from these glands when treated as a serum in a serum-virus neutralization test inhibited fixed virus

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A MEMBRANE FILTER METHOD FOR THE DEMONSTRATION OF BACTERIA BY THE FLUORESCENT ANTIBODY TECHNIQUE

1 A Methodological Study

By

DAN DANIELSSON

Received 29 x 64

Demonstration of bacteria in a water sample by means of the membrane filter method can be done, after filtration, by direct microscopy and staining of the filter This method permits a total count of the trapped organisms to be made, but it gives no information about the kind of organisms (5, 7, 9) The filter can also be placed on a suitable selective medium After incubation in a thermostat, the bacterial colonies grown can usually be indentified Many technical variations of this type of investigation exist (11) Suitable colonies can also be picked out for further biochemical and serological studies These procedures, however, are both laborious and time-consuming

The fluorescent antibody technique seems to offer new possibilities of shortening the time required for bacteriological diagnosis of organisms trapped on a membrane filter *Carter & Leise* (1) gave a brief account of observation of 12 hour old colonies of *Bacillus anthracis* on non fluorescent membrane filters with a low-power dissecting microscope after the colonies had been stained with fluorescent antiserum

The purpose of the present investigation was to test more extensively the possibilities and limitations of demonstrations of bacteria on a non-fluorescent membrane filter It was considered of particular interest to ascertain whether the method could be used for demonstration of small quantities of bacteria

MATERIAL AND METHODS

A *Test Organisms*

An enteropathogenic *Escherichia coli* strain belonging to the serological type 026 B6 and *Shigella guanabara* (serologically related to *E coli* 0112 ac) (4, 8) were used as test organisms

This investigation was supported by grants from the *Delegation for Applied Medical Defence Research Ministry of Defence Stockholm Sweden*

Rabbits guinea pigs, white mice, and hamsters were susceptible to infection by different routes of inoculation Hamsters were found to be more susceptible than white mice for primary isolation Rabbits were more refractory than the other species studied

In addition to various types of cytoplasmic inclusion bodies intra nuclear inclusions were demonstrated in nerve cells of some brains of laboratory infected animals

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E. Fluorescence Microscopy

by fluorescent antibodies was calculated with the equation $X = \frac{N \times R}{20 \times r^2}$, N = the number of bacteria in 20 fields of vision, R = 40 mm or 20 mm (diameter of the filtering surfaces of membrane filters used), r = 0.32 mm (diameter of the field of vision) X = N × 781.25 for filters of 40 mm diameter and X = N × 195.31 for filters of 20 mm diameter

RESULTS

Experiments were first performed to detect bacteria by fluorescent antibodies on the usual white membrane filters This could not be done, however, because of the strong auto-fluorescence of such filters, as well as the difficulty of passing ultraviolet blue light through them

The use of non fluorescent membrane filters permitted detection of single bacteria by fluorescent antibodies (Fig 1), by using incident illumination with the technique described above

TABLE 1

Recovery of Bacteria on Non Fluorescent Membrane Filters Identified by Means of Fluorescent Antibodies

Specimen no	Volume of tap water investigated litre	No of test bacteria added according to		Recovery of test bacteria on non fluorescent membrane filters by fluorescent antibodies	
		Total count X10⁶	Viable count X10⁶	In relation to total count %	In relation to viable count %
1	1	4	2.92	63	85
2	1	4	3.20	79	99
3	2	3.70	3.92	66	63
4	2	4	3.80	86	90
5	1	2	1.60	80	100
6	1	2	1.50	69	91
7	2	2	1.64	61	75
8	2	1.50	1.60	89	84
9	1	1	0.76	76	101
10	1	1	0.80	76	95
11	2	1	0.82	68	84
12	2	0.76	0.80	100	95

Table 1 shows the results of some of the experiments made to study the recovery with the method It can be inferred from this table that the recovery of the test organisms by direct count on non fluorescent membrane filters ranged from 61–100 per cent of the total number of added test bacteria counted with a Burker chamber The range was 63–

The organisms were maintained on glucose agar slopes and subcultures for use were grown in serum glucose broth for 16–18 hours at 37° C

The number of test bacteria added to test samples was estimated as follows One *ml of a 16–18 hours' broth culture of the test strain in question was well mixed* with 9 ml of distilled water (sterilized), and a serial dilution made in 8 tubes with 10 fold dilutions The bacterial density per ml in dilution 10^{-2} was estimated with a Burker counting chamber (depht 0 01 mm) Ten × 10 CD rectangles were counted and the total count calculated from these values The viable count was estimated by inoculating 4–10 Endo agar plates from the dilution 10^{-6} The colonies were counted after 18–24 hours' growth at 37° C The standard error of the mean ranged from 8 20 per cent for the total count, and from 4 12 per cent for the viable count

B *Contamination of Water Samples and Nutrient Broth with Test Bacteria and Filtration Procedures*

The desired number of the relevant test bacteria was added to volumes of 1 or 2 litres of tap water The bacteria were mixed in the water samples by stirring on a magnetic stirrer for 1 hour at 3 4° C The samples were then filtered through non fluorescent membrane filters (Millipore HAB (P)G 047 A pyrex filter holder with a 250 ml funnel was used for this purpose and a differential pressure of 50–60 mm Hg was applied for filtration Filtration of 1 litre was completed within 2 minutes and of 2 litres within 4 minutes The membrane filters were then stained with fluorescent antibodies as described under D

In experiments with mixed bacteria the two test strains used—*E coli* 026 and
broth to a total number of
ted at 37° C on a magnetic
6 7, 10 and 13 hours and
ipore HAB (P)G 025) The
nt antibodies and counted
was estimated by plating
counted after 24 hours at
since *Shig guanabara* did
not ferment lactose Serological tests were included as controls

C *Productions of Antiserum and Conjugation with Fluorescent Substance*

Rabbit antisera specific to enteropathogenic *E coli* 026 B6 and *Shig guanabara* were prepared according to the directions of *Edwards & Ewing* (6) The globulin portion of each antiserum was obtained by precipitation in cold with ammonium sulphate at half saturation followed by labelling with fluorescein isothiocyanate (12) using a slightly modified procedure (2) Unconjugated material was removed by passing the conjugate through a column packed with Sephadex G 25 (Pharmacia) in 0 01 M phosphate buffered physiological saline pH 7 2 (abbreviated to PBS) The conjugate was then filtered through an HA membrane filter (Millipore)

D *Staining of Membrane Filters and Ordinary Smears with Fluorescent Antibodies*

Organisms trapped on non fluorescent membrane filters were stained in the following way After filtration of a water sample through an HAB (P)G 047 membrane filter ,or of a broth sample through an HAB (P)G 025 membrane filter circular pieces 12 5 mm in diameter were stamped out with a metal die They were placed on a metal supporting screen in a Swinny hypodermic adapter (Millipore) closed at the outlet and filled with PBS up to the screen A few drops of the conjugated antiglobulin were placed on the filter and the staining was carried out for 45 60 minutes The outlet in the Swinny adapter was then opened the filter was washed with PBS and mounted on a slide To prevent drying the slide was placed in a humid chamber at 3 4° C until used

Staining of ordinary smears with the conjugated antiglobulin was performed in the customary way

It was found that the conjugates used to stain bacteria on non fluorescent membrane filters should have a high titre For this purpose they could be diluted only 1 18–1 16 when they gave a staining titre of 1 128 1 256 in ordinary smears

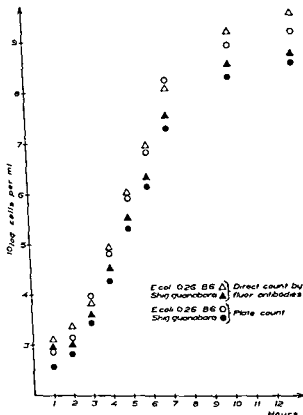


Fig 2

Simultaneous growth of *E coli* O26 B6 and *Shig guanabara* in ordinary broth Number of organisms estimated by direct count on non fluorescent membrane filter and by plate count (*cf* text)

within one hour by means of the fluorescent antibody method Since this method permitted a specific serological diagnosis, it had definite advantages over direct identification of microorganisms on white membrane filters with the ordinary microscope technique It did however, require the fluorescence microscope to be equipped for incident illumination, and the conjugate must have a relatively high staining titre if reproducible results were to be obtained

Jannasch (9) *Ehrlich* (7), and *Ecker & Lockhart* (5) showed that direct microscopy of white membrane filters, on which microorganisms from water samples or broth cultures had been trapped and stained by ordinary dyes permitted rapid quantitative estimation of the total number of bacteria It could be demonstrated in the present study that the fluorescent antibody method, in combination with the membrane filter technique, also allowed quantitative determination of the bacteria identified serologically In this connection it was of interest to note



Fig 1
Enteropathogenic *E coli* 026 B6 trapped on a non fluorescent membrane filter and stained with FITC conjugated anti 026 B6 globulin
Photographed on Kodak Tri X at a microscopic magnification of 400 X exposure time 40 sec

101 per cent applied in a comparison with the viable count It was found that the results were largely the same irrespective of whether the bacteria were suspended in one or two litres In experiments in which the recovery was relatively low (about 60–75 per cent) the filtrate was refiltered to study whether any bacteria had passed the filter No additional growth was obtained It was found, however, that when the bottles used in these experiments were washed an extra time bacteria could be grown from these washings amounting up to 0,1 per cent–2 per cent of the originally added test bacteria

Fig 2 shows the results of an experiment in which comparative counts of the two test strains growing simultaneously in nutrient broth were made by direct count on non-fluorescent membrane filters after staining with fluorescent antibodies, and by viable count It can be seen that the direct count, as a rule, gave a somewhat higher value than the viable count in broth strains The direct count of each sample was completed within 2 hours, whereas the viable count took 18–24 hours

DISCUSSION

Bacteria suspended in tap water or cultured in nutrient broth and then trapped on non-fluorescent membrane filters, could be indentified

10 *Miller E J* A method for the removal from membrane filters of micro organisms filtered from water and air J appl Bact 26 211 1962
11 *Standard methods for the examination of water and wastewater* Eleventh edition 1960 American Public Health Ass Inc New York A
12 *Riggs J L Seinwald R J Burckhalter J Downs Cora M & Metcalf T G* Isothiocyanate compounds as fluorescent labelling agents for immune serum Am J Path 34 1081 1958

that on investigation of broth cultures consistently higher numbers of bacteria were obtained with the fluorescent antibody method than with a viable count In our experiment a mixed culture of two strains of bacteria was used It must, however, be pointed out that some of the microorganisms demonstrated in a direct count were present in pairs or short chains It is questionable whether they had been capable under such circumstances of forming distinguishable colonies Another possible explanation is that certain of the bacteria identified by the fluorescence method might not have been viable as a similar trend is observed by comparing the count of bacteria in Burker chamber with the viable count

In the present study, the recovery of the fluorescent antibody method have good reproducibility in bacterial concentrations of 100,000 bacteria per litre In concentrations below 1000 bacteria per litre, demonstration of the microorganisms, however, proved to be a somewhat time-consuming process It seems possible to overcome this drawback by the use of an enrichment procedure, which permits very small numbers of bacteria to be identified within a few hours This modification will be described in a later paper (3)

SUMMARY

Bacteria suspended in tap water or cultured in broth, and then trapped on non-fluorescent membrane filters, could be identified within one hour by means of the fluorescent antibody method For this purpose the fluorescence microscope was equipped for incident illumination The technique described allowed a quantitative determination of the bacteria identified serologically The limitations of the sensitivity of the method were discussed

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4 *DeAssis A* Shigella guanabara tipo serologico destacado do grupo do B cey [illegible]
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the white filters was placed on a Conradi Drigalski blue agar plate and incubated at 37° C Colonies grown after 18 24 hours were then tested by current biochemical and serological procedures The remaining white filter was put into a conical Pyrex flask and washed with 10 ml of saline for 15 20 minutes the process being assisted by the use of glass beads (4) The saline was then centrifuged for 20 minutes at 3000 r p m The supernatant was decanted and the sediment suspended in a small volume of saline from which smears were made and stained with fluorescent anti bodies in the usual way From the black non fluorescent membrane filter circular pieces 12 5 mm in diameter were stamped out with a metal die They were then stained with fluorescent antibodies

b) *Model 2* The test organisms used were *E coli* 026 B6 alone or together with an equal number of *Shig guanabara* They were added to tap water in concentra tions ranging from 5000 2/litre of water One-litre samples were filtered through white membrane filters (Millipore HAW (P)G 047 One filter was placed on a Con radi Drigalski blue agar plate and incubated at 37° C. Colonies grown after 18 24 hours w
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A Swinny hypodermic adapter (Millipore) attached to a standard Luer lock hypo dermic syringe was used for this purpose The organisms trapped on the filter were then stained with fluorescent antibodies This technique was denoted as the "two step procedure" Finally samples of about 10 ml were centrifuged at the same intervals as above for 20 minutes at 3000 r p m The broth was decanted and the sed ment suspended in a small volume of saline Smears were made and stained as previously described

c) *Model 3* The test organism *E coli* 0119 B14 was added to tap water in con centrations of 200–300 bacteria per litre and the other two test strains—*E coli* 0°6 B6 and *Shig guanabara*—were added to the same samples in concentrations ranging from 50–5 bacteria per litre Filtration procedures and enrichment of the filters were the same as those described for model 2

C. *Fluorescent Antibody Procedures*

The preparation of antisera conjugation with fluorescent substance and staining of smears and filters as well as the microscope equipment were the same as in the previous study (1) The number of bacteria detected by fluorescent antibodies on ordinary smears and non fluorescent membrane filters was counted in 10–40 fields of vision At least 50 fields were examined before a specimen was regarded as negative

RESULTS

The results of the experiments according to model 1 are listed in Table 1 It can be seen that the non fluorescent membrane filter permitted identification of the test organisms within one hour Reproducible values could be obtained to a minimum concentration of about 1000 bacteria per litre of water When the bacteria were eluted from ordinary white membrane filters, 2 hours were required for a diagnosis The lower limit of reproducibility was then about 5000 bacteria per litre of water With both types of filter, a large number of microscopical fields had to be inspected for demonstration of the bacteria at the lower concentrations

The results of the experiments according to model 2 are shown in Fig 1 It can be inferred that when the relevant test organisms were present in concentrations of 50–2 per litre of water in the original sample they could—after enrichment and concentration by centrifugation—be demonstrated with fluorescent antibodies on ordinary

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A MEMBRANE FILTER METHOD FOR THE DEMONSTRATION OF BACTERIA BY THE FLUORESCENT ANTIBODY TECHNIQUE

2 *The Application of the Method for Detection of Small Numbers of Bacteria in Water*

By

DAN DANIELSSON and GUNNAR LAURELL

Received 29 x 64

Non-fluorescent membrane filters can easily be adapted for immunofluorescent identification of bacteria in a water sample trapped on such a filter A suitable method for this purpose has been described in an earlier paper (1) The method was found to permit the diagnosis of the type of bacteria within one hour, as well as quantitative estimation of the number of bacteria in a sample Small numbers of bacteria (< 1000 bacteria per litre), however, were gradually more difficult to identify and the method therefore had a certain quantitative limitation An account is given in the present paper of further tests of the method with respect to sensitivity and the time required for the identification of the bacteria

MATERIALS AND METHODS

A *Test Organisms*

The test organisms consisted of two enteropathogenic strains of *Escherichia coli* belonging to the serological types 026 B6 and 0119 B14 in addition to *Shigella guanabara* (serologically related to *E coli* 0112 ac (see *De Assis* (2) and *Ewing & Kauffmann* (3))

B *Contamination of Tap Water with Test Organisms Filtration Procedures and Handling of Membrane Filters*

The test bacteria were counted as described earlier (1) and suspended in samples of tap water which were then filtrated through membrane filters Three different experimental models were used

a) *Model 1* The test strain *E coli* 026 B6 was added to samples of tap water in bacterial concentrations of 100 000 per litre to 100 per litre Two of three 1 litre samples, each containing the same number of microorganisms were filtered through separate white membrane filters (*Millipore HAW (P)G 047*) and the third sample through a black non fluorescent membrane filter (Millipore HAB(P)G 047) One of

This investigation was supported by grants from the *Delegation for Applied Medical Defence Research Ministry of Defence Stockholm Sweden*

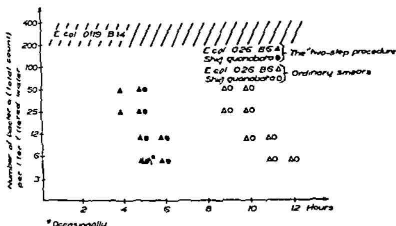


Fig 2

... of small quantities ... ative domination of ... white membrane filters treated as described for model 2 (see text!)

The experiments according to model 3 are shown in Fig 2 It will be seen that both *E coli* 026 B6 and *Shig guanabara* were demonstrable within 4–6 hours with the two step procedure, and after 9–12 hours with ordinary smears The demonstration of the test organisms was not disturbed by the occurrence of an irrelevant organism in relatively high concentrations

DISCUSSION

Experiments with the aim to demonstrate small numbers of bacteria in water by means of fluorescent antibodies have been continued and extended It could be shown that the lower limit of sensitivity for direct microscopy, after elution of bacteria from white membrane filters, is about 5000 per litre of water By direct staining of non-fluorescent membrane filters, the lower limit could be reduced to 1000 bacteria per litre Both methods permitted bacteriological identification after 1 2 hours Even if the time for diagnosis could be regarded as satisfactory, it seemed desirable to increase the sensitivity A "two step procedure" was tested for this purpose With this technique the bacteria are first trapped on an ordinary white membrane filter After elution and incubation on a suitable substrate for varying periods samples are refiltered through non-fluorescent membrane filters, which are then stained in the usual way With this technique, the limit of sensitivity could be decreased to 50–2 bacteria per litre of water This, however, did imply a prolongation of the time required for diagnosis to 4–6 hours Despite this prolongation, the method can

smears after 8–12 hours. This implies a gain in time of 24–30 hours as compared to conventional culture and serological determination. Still more time could be gained by using the two-step procedure described. Thus, the test organisms in question could be demonstrated with this procedure within 4–6 hours, when they were present in the original sample in concentrations of 50–2 per litre. As a rule, the test strain *E. coli* 026:B6 was demonstrated 1 hour earlier than the test strain *Shig. guanabara*.

TABLE 1

Identification of E. coli 026: B6 in Varying Concentrations trapped on non-fluorescent and on White Membrane Filters

Number of bacteria per litre	Number of bacteria identified on			
	Non-fluorescent membrane filters		Ordinary smears after washing of white membrane filters	
	Numbers of bacteria	Numbers of fields of vision counted	Numbers of bacteria	Numbers of fields of vision counted
100 000	30–40	10	1–5	10
10 000	4–8	10	1–3	40
5 000	2–8	20	1–2	40
1 000	approx. 1–2	40	<1	50
100	<1	50	<1	50
Time for diagnosis	1 hour		2 hours	

Fig. 1

Number of hours for demonstration with fluorescent antibodies of small quantities of *E. coli* 026: B6 and *Shig. guanabara* concentrated on ordinary white membrane filters treated as described for model 2. (See text.)

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ON THE EFFECT OF PENICILLIN IN THE PRODUCTION OF AUXOTROPHIC MUTANTS OF *NEISSERIA MENINGITIDIS*

By

SVERRE LIF

Received 3 x 64

Biochemical deficient mutants in a bacterial culture can only be isolated through plating and examination of single colonies After treatment with various mutagens this is a possible although laboursome technique and was employed in the beginning of the era of microbial genetics (12) The proportion of such mutants in a culture can obviously be greatly increased with an agent selectively killing the wild-type cells The discovery (6) that penicillin will kill only actively dividing cells led to the successful employment of this agent in the selection of biochemical mutants of bacteria Such a technique was independently described by *Davis* (2), and by *Lederberg & Zinder* (13) in 1948 The procedure involves Exposure of the cells to the action of a mutagenic agent, growth of the treated cells until phenotypic expression of the induced mutations and finally, growth in minimal medium in the presence of penicillin for about 6–24 hours (9) An improvement of this technique was introduced by *Gorini & Kaufman* (4) In this procedure the cells are allowed to reach the logarithmic growth phase before penicillin exposure, and the exposure time is kept short enough to prevent any lysis of killed cells The latter point is of importance since such lysis might release into the growth medium factors that will enable the mutant cells to start growing with subsequent killing by the antibiotic

In this laboratory the production of auxotrophic mutants of *N meningitidis* has been achieved by a modification of the penicillin technique of *Gorini & Kaufman* using ultraviolet (UV) light as the mutagenic agent (8) The yield of mutants, however, was consistently very small and it became pertinent to analyse the effects of each of the steps in the procedure The mutagenic effect of UV light on meningococci is reported elsewhere (14), the activities of penicillin as a selective agent is the subject of the present report

be regarded as satisfactory with respect to sensitivity, as well as to the time marginal for a diagnosis

In this work equipments were used which easily allowed filtration of tap water samples of one litre and 2 bacteria per litre was the lowest concentration tested It might be possible, however, to increase the sensitivity of the method still more by using equipments allowing mechanical concentration of small numbers of bacteria in larger volumes of water, *e g* by continuous flow centrifugation

It could also be demonstrated that the method functions well for mixed cultures, in which an irrelevant microorganism dominates in relation to that to be diagnosed It must be pointed out, however, that the tests were made on experimentally infected tap water It remains to be shown whether it is applicable to naturally more or less polluted water

SUMMARY

The limit of sensitivity for demonstration of small quantities of bacteria in water by means of the fluorescent antibody method in combination with the membrane filter technique was investigated After elution of bacteria from white membrane filters the lower limit of sensitivity for direct fluorescence microscopy was about 5000 per litre of water By direct staining of non fluorescent membrane filters, the lower limit could be reduced to 1000 bacteria per litre, and by combining this technique with an enrichment procedure, it was possible to demonstrate bacteria present in a concentration of 2–50 per litre within 4 6 hours

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ON THE EFFECT OF PENICILLIN IN THE PRODUCTION OF AUXOTROPHIC MUTANTS OF *NEISSERIA MENINGITIDIS*

By

SVERRE LIF

Received 3 x 64

Biochemical deficient mutants in a bacterial culture can only be isolated through plating and examination of single colonies After treatment with various mutagens this is a possible although laboursome technique and was employed in the beginning of the era of microbial genetics (12) The proportion of such mutants in a culture can obviously be greatly increased with an agent selectively killing the wild-type cells The discovery (6) that penicillin will kill only actively dividing cells led to the successful employment of this agent in the selection of biochemical mutants of bacteria Such a technique was independently described by *Davis* (2), and by *Lederberg & Zinder* (13) in 1948 The procedure involves Exposure of the cells to the action of a mutagenic agent, growth of the treated cells until phenotypic expression of the induced mutations and finally, growth in minimal medium in the presence of penicillin for about 6–24 hours (9) An improvement of this technique was introduced by *Gorini & Kaufman* (4) In this procedure the cells are allowed to reach the logarithmic growth phase before penicillin exposure, and the exposure time is kept short enough to prevent any lysis of killed cells The latter point is of importance since such lysis might release into the growth medium factors that will enable the mutant cells to start growing with subsequent killing by the antibiotic

In this laboratory the production of auxotrophic mutants of *N meningitidis* has been achieved by a modification of the penicillin technique of *Gorini & Kaufman* using ultraviolet (UV) light as the mutagenic agent (8) The yield of mutants, however, was consistently very small and it became pertinent to analyse the effects of each of the steps in the procedure The mutagenic effect of UV light on meningococci is reported elsewhere (14), the activities of penicillin as a selective agent is the subject of the present report

MATERIALS AND METHODS

Bacterial strains Auxotrophic mutants of N *meningitidis* strain M1 were provided by *Jyssum* (8) Both transformable and non transformable strains were used In comparative experiments with *E coli* biochemical mutants of strain 635 and K 12 T71 were employed (5)

Media and antibiotics Heart infusion broth (HIB) was used as the complete liquid medium Solid complete medium was either HIB solidified with 1 5 per cent agar (Difco) or blood agar plates The basal medium of meningococci has been described by *Jyssum* (8) and that of *E coli* by *Davis & Mingioli* (3) Penicillin was used in the form of benzyl penicillin G (Glaxo) Ampicillin (α aminobenzylpenicillin) was obtained through the courtesy of Norsk Astra A/S Penicillinase was obtained from Difco ('Bacto penase)

Cultural techniques For the production of actively dividing and resting cells in minimal medium a biochemical mutant was cultivated overnight in basal medium supplemented with the appropriate growth factor The next day the cells were spun down washed once in saline and finally resuspended in basal medium The suspension was diluted 1 10 in basal medium into two tubes with the growth factor added to only one of the tubes Growth was followed either by a Beckman model C colorimeter (absorption at 524 mμ) or by measuring the increase in viable cell count Incubation took place at 37° C in a humid atmosphere containing approximately 10 per cent CO_2 (7)

Deoxyribonuclease (DNase) was obtained from L Light & Co Ltd and was dissolved 10 mg per ml in sterile 2 per cent gelatine solution (stock solution) Before use 1 part stock solution was mixed with 4 parts 2 M $MgCl$ and 5 parts 2 per cent gelatine solution

RESULTS

The effect of penicillin on actively dividing and resting cells was followed both by measuring the decrease in viable cell count and in the optical densities of cultures exposed to 2000 units penicillin per ml Typical inactivation curves of growing and resting cells of *N meningitidis* and *E coli* are presented in Fig 1 and Fig 2 The strain of meningococcus used in Fig 1 is a histidine-requiring, competent strain Identical curves were obtained with a threonine requiring mutant and with incompetent variants of both mutants During active division,

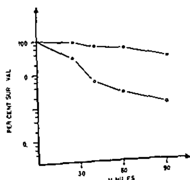


Fig 1
Inactivation by 2000 units penicillin G of growing (—○—○—○—○—) and resting (—●—●—●—●—) cells of N *meningitidis*

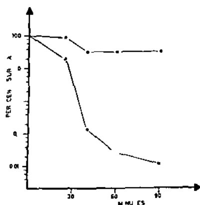


Fig 2
Inactivation by 2000 units penicillin G of growing (—○—○ ○—) and resting (—●—● ●—●—) cells of *E coli*

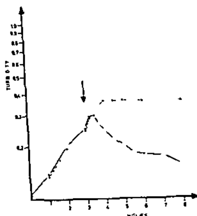


Fig 3
Penicillin induced lysis of *N meningitidis*
Arrow indicates the time of addition of penicillin (2000 units per ml)
— —×—×—×— Lysis in basal medium
—●——●——● Lysis in basal medium containing 0 08 M Mg⁺⁺

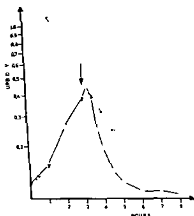


Fig 4
Penicillin induced lysis of *E coli*
Arrow indicates the time of addition of penicillin (2000 units per ml)
— — — — — Lysis in basal medium
—●——●——● Lysis in basal medium containing 0 08 M Mg⁺⁺

approximately 99 per cent of the colony-forming units are killed during the first 2 hours of exposure Almost no further inactivation can be detected, and even after 4 hours of exposure the titre has seldom decreased by a factor of more than 10" When meningococcal cells were grown in complete medium, however, a much more rapid killing took place with a sterilization of the culture within 3–4 hours

The strain of *E coli* used in Fig 2 is a threonine-requiring mutant of K 12 T 71 A rapid killing is observed and the culture is regularly

sterilized within 4 hours The viable count of resting cells of either species was only slightly reduced by penicillin

The great resistance to the action of penicillin exhibited by meningococci in basal medium was not caused by the destruction of penicillin Filtrates of cultures treated with penicillin for 24 hours still possessed great antibacterial activities Furthermore, the medium itself did not have any detectable antagonistic effect on the penicillin action Cells of *E coli* growing in this medium exhibited the same degree of sensitivity to the drug as when they were grown in their own minimal medium

The penicillin effects were also followed by measuring the changes in optical densities, a method recently advocated by *Boman & Eriksson* (1) Suspensions of non-dividing cells did not exhibit any change in optical densities upon the addition of penicillin In active division, however, there were striking effects as reported in Figs 3 and 4 In spite of an approximately similar growth rate at the time of penicillin addition, there is much more pronounced lysis in the *E coli* culture than in the meningococcal one

DNase in a concentration of 100 μg per ml was originally included in some experiments in order to prevent any transformation of mutant cells back to prototrophy by DNA released from penicillin-killed wild type cells A lysis-inhibition was then observed which upon further examination turned out to be caused, not by the enzyme itself, but by the Mg^{++}ions added simultaneously to ensure enzymatic activity (15) In Fig 3 and Fig 4 results have been presented from experiments in which Mg^{++} (0 08 M) is present in the medium

The final concentration of 0 08 M Mg^{++} completely prevented any lysis of meningococcal cells while it greatly reduced the lysis of cells of *E coli* This lysis-preventing effect of Mg^{++}ions is previously known and has been employed in experiments designed to produce protoplasts of bacteria by means of penicillin (10, 11)

From the experiments described, it may be concluded that the elimination of growing cells in basal medium by penicillin is much less pro-
... in *E coli* cultures In search of a more
... test the effects of ampicillin (α amino-
... c has been reported to have a rather
high bactericidal effect on Gram-negative organisms (17) It was used as the D-isomer in concentrations of 40 μg per ml, and the effects on meningococci were determined both by measuring the decrease in viable cell count and by the decrease in optical densities The results, however, did not reveal any differences in the degree of activity exhibited by the two penicillins in meningococcal basal medium

DISCUSSION

From the results presented it can be concluded that the bactericidal action of penicillin in basal medium is less pronounced on cells of

N meningitidis than on cells of *E coli* This is evident from the decrease in viable cell count, from the extent of penicillin-induced lysis, and from the lysis protecting effect of 0 08 M Mg^{++}ions This difference can not be explained on the basis of higher growth rates of *E coli*, as growth curves measured by optical methods (Figs 3 and 4) do not reveal any significant difference between the two species Working with *B subtilis*, *Nester* (16) found that populations of highly competent cells were less susceptible to the action of penicillin than less competent ones Such a difference could not be detected in the strains of *N meningitidis* employed in the present investigation

One possible explanation of the low bactericidal effect of penicillin on cells of meningococci could be the great heterogeneity and interdependence that is established between cells when grown in minimal medium It has been postulated (8) that in such a growing population only a fraction of the cells is active in the biosynthesis of certain essential metabolites, while the rest grow by cross feeding This will for instance explain why only a fraction of the cells is able to give rise to a colony when plated on minimal agar, since the derepression or induction of the required enzymes might not take place under such conditions (8) The same hypothesis might account for the low bactericidal effect of penicillin observed in minimal medium When the drug is added to such a culture, only the most actively metabolizing cells will be killed The part of the population which is cross fed by these cells will stop to grow and consequently remain unaffected by the presence of penicillin That the cells are genotypically sensitive to the action of the drug is indicated by the rapid killing that takes place when the cells are grown in complete medium

The data in Fig 1 show that penicillin has a small selective effect on cells of meningococci in basal medium since there is almost no killing of resting cells However, the difference presented in Fig 1 must be considered to be maximal as the two inactivation curves are determined in separate tubes When mutant cells and wild type ones are present in the same population one might very well imagine that there may be a considerable metabolic activity of the former as a result of cross-feeding Such cross feeding is indeed so pronounced in this species that when two distinct, auxotrophic incompetent mutants are plated in mixture on solid, basal medium with no growth factors added, colonies appear that are composed of both mutant types but with no prototrophic recombinants (8) This would then indicate that when penicillin is added to a mixture of wild type and mutant cells growing in minimal medium, there might also be a considerable killing of the latter Some preliminary experiments performed with artificially mixed cultures have indeed supported this assumption It is concluded, therefore that the selective effect of penicillin in the production of biochemical mutants of *N meningitidis* is of small value

SUMMARY

The bactericidal effect of penicillin on growing and resting cells of meningococci has been studied In comparative experiments strains of *E coli were employed In complete medium the cells of both species* were rapidly inactivated by penicillin, but in minimal medium actively dividing meningococcal cells exhibited a much greater resistance than actively dividing cells of *L coli* No difference was observed between the activities of penicillin G and ampicillin (*α* aminobenzylpenicillin) under the experimental conditions used Some possible explanations of the observations are given

It is concluded that the use of penicillin in the isolation of biochemically deficient mutants of *N meningitidis* is of small value

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PRODUCTION OF AUXOTROPHIC MUTANTS OF *NEISSERIA MENINGITIDIS* BY NITROUS ACID

By

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Received 16 x 64

After the formula of a minimal medium of meningococci was given (7) it became possible to isolate biochemically deficient mutants of this organism Earlier reports from this laboratory (8) have described such isolations utilizing the mutagenic effect of ultraviolet (UV) light with subsequent concentration of induced mutants by the modified penicillin technique of *Gorini & Kaufman* (4) The yield of mutants was very low, however, and more extensive investigations on the effect of UV light and penicillin were performed Under the conditions employed, it became clear that the mutagenic effect of UV light on meningococci was hardly detectable (14) Furthermore, the selective killing effect of penicillin on growing and non-growing cells of meningococci in minimal medium is much less pronounced in this species than in *E coli* (15)

These results clearly indicated the need of finding a more effective mutagen for the production of biochemically deficient mutants of meningococci This paper reports some experiments in which the effect of nitrous acid has been examined This compound was first shown to be a strong mutagen by *Mundry & Gierer*, using tobacco mosaic virus and its RNA (18, 3) Many biological systems have been shown to react by mutation when exposed to this compound These include the DNA-containing bacteriophages (23, 20), animal viruses (5, 21), transforming principle (16, 6, 17), and bacteria (9, 10) The effect of nitrous acid is probably due to deaminations of the bases adenine, cytocine, and guanine which give the deamination products hypoxanthine, uracil and xanthine, respectively (19) Such base changes will introduce errors in the base pairings taking place during DNA-replication, and a mutation may result (2)

Working with *E coli*, *Kaudewitz* (9, 10) could isolate around 4 per cent auxotrophic mutants after having treated the cells with nitrous acid to a survival of 10^{-5} The high mutation frequency and the fact that no penicillin treatment was involved in the procedure, initiated the present study Some experiments with *E coli* are included for comparison and control

MATERIALS AND METHODS

Bacterial strains A histidine requiring strain of meningococci easily transformed by DNA (strain 12 his) has previously been described (8) *E coli* K 12 T71 has previously been used in this laboratory (14)

Media Heart infusion broth (HIB) and agar (both from Difco) were used as liquid and solid complete media The modified minimal medium of meningococci has been developed by *Jyssum* (8) and that of *E coli* described by *Davis & Mingioli* (1) Amino acids pyrimidine and purine bases were added to a final concentration of 100 µg/ml in the medium of *E coli* and to 50 µg/ml in the medium of meningococci Vitamins were added to final concentrations of 10 µg/ml in both minimal media

Culture techniques Cells to be exposed to nitrous acid were grown in either complete or minimal media After the desired *period of incubation* the cells were *spun* down in the cold (+4° C) resuspended in saline and incubated various times at 37° C before starting the treatment with nitrous acid

Nitrous acid treatment The procedure and media employed are in general as those described by *Kaudewitz* (10) Phagenpuffer 10 ml 0 1 M $MgSO_4$ 10 ml 0 01 M CaCl 1 ml 1 per cent gelatine 7 4 g NaH_2PO_4 12 H_2O 1 5 g KH_2PO_4 4 g NaCl 5 g K_2SO_4 H_2O ad 1000 ml.

Standard reaction mixture consisted of the following three components

1 vol bacterial suspension
1 vol NaNO solution
1 vol acetate buffer 0 6 N

The concentration of NaNO and the pH of the acetate buffer employed will be described in the result section The reaction took place in a 37° C waterbath and was stopped by diluting at least tenfold in cold (+4° C) Phagenpuffer The cells were plated in appropriate dilutions on complete medium or when necessary concentrated by centrifugation before plating

Detection of auxotrophic mutants was accomplished by using the velvet replica technique of *Lederberg & Lederberg* (13) Colonies that appeared on complete medium after nitrous acid treatment were replicated on the minimal medium of the parental strain Colonies that did not grow on the latter were further isolated and the growth requirements determined by a method of *Kaudewitz et al* (12) Since heavy inocula are needed to start growth of meningococci on minimal media (7) 1 per cent HIB was added to the basal medium of this organism before replica plating This did not interfere with the detection of auxotrophic mutants as they only gave a very faint background growth on this concentration of HIB The growth that did take place however was sufficient to ensure full growth of all the replicated wild type colonies

RESULTS

Effect of Nitrous Acid on E coli

In the reaction mixture used (0 017 M $NaNO_2$ at pH 4 3) the number of viable cells decreased by a factor of 10 every second minute At various levels of survival colonies were tested for their ability to grow on minimal medium Nitrous acid turned out to be a potent mutagen inducing mutations to prototrophy in high frequencies The number of auxotrophic mutants among the surviving cells was in each experiment found to depend upon the length of exposure to nitrous acid This is in agreement with the result of *Kaudewitz* (10) However as much as 4 per cent auxotrophs was never found In six independent experiments with a surviving fraction ranging from 10 3 to 10 the percentage of auxotrophic mutants varied from 0 14 to 1 6 In these experiments 29 mutants were isolated out of a total of 9 298 colonies examined (aver

age 0 31 per cent) Table 1 shows the distribution of growth factors among these mutants

TABLE 1

Distribution of Nutritional Requirements of 29 Nitrous Acid Induced Auxotrophic Mutants of E coli

Adenine	3	Lysine	1
Guanine	1	Methionine	1
Uracil	3	Proline	1
Cysteine	1	Tryptophane	1
Glycine	1	Nicotinamide	2
Glycine or Serine	2	Thiamine	1
Histidine	1	Other unanalysed requirements	10

Effect of Nitrous Acid on N meningitidis

Preliminary experiments showed that in contrast to *E coli* cells, meningococci were very rapidly inactivated when exposed to acid solutions Acetate buffers (0 6 N) of pH 4 3, 4 7, 5 0, and 5 5 were prepared, and the rate of inactivation of meningococci in these solutions determined After five minutes of incubation the number of viable cells had decreased by factors of 10^4 in buffer pH 4 3, 10^3 in buffer pH 4 7, 10^2 in buffer pH 5 0 In buffer pH 5 5 no significant inactivation could be detected even after 15 minutes of incubation To obtain an inactivation specifically due to the action of nitrous acid, buffer pH 5 5 was used in all subsequent experiments In order to obtain an inactivation rate somewhat comparable to that of *E coli*, the concentration of $NaNO_2$ had to be increased from 0 017 M to 0 33 M The standard reaction mixture of meningococci was therefore

1 vol bacterial suspension
1 vol $NaNO_2$ 1 M
1 vol acetate buffer 0 6 N, pH 5 5

The inactivation curve obtained from meningococci grown in minimal medium when they were exposed to such a reaction mixture has been presented in Fig 1 Cells grown in complete media exhibited greater sensitivity both to acid pH and to the action of nitrous acid, and minimal medium was therefore mainly used Experiments with *E coli* in the meningococcal reaction mixture gave almost no inactivation during the first ten minutes of incubation

In samples representing various fractions of surviving units, colonies were tested for induced nutritional requirements Auxotrophic mutants were regularly isolated although in a frequency only about one fifth of that obtained with *E coli* As only a few mutant colonies were isolated in each experiment no accurate determination of the relationship between mutation induction and length of [illegible]

[illegible] n both in complete and minimal media

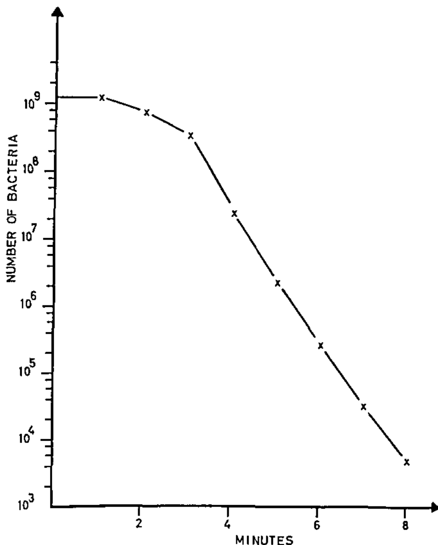


Fig 1

Inactivation curve of cells of N *meningitidis* grown in minimal medium and exposed to 0 33 M nitrous acid (pH 5 5)

Such differences in cell physiology did not significantly influence the yield of mutants From 24 independent experiments performed 20 biochemically deficient mutants were isolated from a total of 34 528 colonies examined (average 0 06 per cent) Table 2 shows the distribution of growth factor requirements of these mutants

TABLE 2

Distribution of Nutritional Requirements of 20 Nitrous Acid Induced Auxotrophic Mutants of N meningitidis

Arginine	1	Proline	1
Cysteine	3	Threonine	1
Glycine	1	Ca pantothenate	1
Leucine	1	Nicotinamide	1
Methionine	1	Thiamine	1
Other unanalysed requirements			8

The replica plating technique used above will only detect pure mutant colonies *Kaudewitz et al* (11) have shown that colonies composed of both wild type and mutant cells regularly appear after nitrous acid treatment When replicated on minimal medium the wild type cells will grow and the induced mutation remain undetected Such colonies are probably formed by a cell harbouring a DNA molecule in which one of the sister strands is mutated giving rise to auxotrophic cells and one is unchanged giving rise to wild type cells The presence of such cells was examined by taking 25 colonies from each of three independent experiments suspending them in saline and plating on complete agar plates in dilutions giving around 100 colonies per plate Subsequent replication of three plates of each original colony (*i e* around 300 cells) upon minimal agar gave no mutants With the low proportion of pure mutant colonies in mind the proportion of mixed colonies was not considered great enough to justify examinations of larger numbers of single colonies this way

DISCUSSION

The results of the control experiments performed with *E coli* are in good agreement with those of *Kaudewitz* (9 10) and will not be further discussed

The greater sensitivity of meningococci to acid solutions necessitated a reaction mixture of 0 33 M $NaNO_2$ at pH 5 5 The inactivation curve of meningococci in this solution is of the typical sigmoidal type with the exponential killing starting after around 3 minutes of incubation *Kaudewitz* (10) analysing the inactivation kinetics of resting cells of *E coli* found a two hit inactivation curve indicating that both strands of the DNA molecule have to suffer a lethal deamination in order to cause inactivation

The extent of the shoulder in the inactivation curve of meningococci suggests that more than two hits are needed for inactivation Possible causes of this phenomenon is the nature of the colony forming unit and the pH of the reaction mixture On the basis of UV light inactivation experiments it has been postulated that the two cells making up the diplococcus are inter dependent when in active division while independent of each other in the resting stage (14) As only resting cells were employed in the present experiments this would predict that at least four hits would be required to inactivate the colony forming unit Experiments with cells in active division could not be performed as the buffering capacities and ions of the growth medium had to be re

is undissociated HNO

curve might therefore

also partly be caused by a slow production of the active compound at

the pH used Any such delay would be reflected by a corresponding delay in the inactivation of the cells

There are several possible causes of the low induced mutation frequency observed with cells of meningococci *Vielmetter & Schuster* (22) found that the deaminations of guanine were mainly lethal, while those of adenine and cytosine were responsible for the mutagenic effect of nitrous acid Furthermore, the rate of deamination of guanine compared to that of adenine and cytosine was much greater at pH 5 5 than at pH below 5 This phenomenon would actually predict the low mutation frequency observed Control experiments with *E coli* in the meningococcal reaction mixture could unfortunately not be performed since the inactivation of such cells in this mixture was almost negligible

The pH of the reaction mixture is most probably the main cause of the low mutation induction effect of nitrous acid on meningococci Other additional causes might be the diplococcal nature of the colony-forming unit The proportion of mixed colonies, for instance, might be expected to be greater in this species than in *E coli* The examination of around 300 cells from each of 75 colonies appearing after nitrous acid treatment revealed no mutant cell and further examinations were not performed In such a diplococcal system the repair of an induced damage might also be very effective In this connection, it is remarkable that the mutagenic effect of UV light on this species is almost negligible (14)

It has been found (17) that the native DNA of *H influenzae* is insensitive to the mutagenic effect of both nitrous acid and UV light This could be explained by postulating that both of these two agents induced cross linkages in the DNA-molecule that would prevent the integration of this part in transformation tests (17) Whether such a process of cross-linkage also exists in meningococci is completely unknown

In spite of all the mentioned disadvantages, however, nitrous acid regularly induces biochemically deficient mutants of meningococci The main disadvantage of the system is that due to the low yield of mutants, the chance of obtaining mutants with blocks in specific pathways is very low

SUMMARY

The inactivating and mutagenic effects of nitrous acid on cells of *N meningitidis* have been studied In control experiments with *E coli*, nitrous acid turned out to be the expected mutagen On the average, 0 31 per cent of the cells surviving nitrous acid treatment gave rise to pure, auxotrophic mutant colonies

The pH 4 2 used in the nitrous acid treatment of *E coli* inactivated the meningococci very rapidly The reaction mixture which gave a con-

vement killing specifically due to nitrous acid consisted of 0 33 M $NaNO_2$ at pH 5 5 The inactivation of meningococci in this reaction mixture followed a sigmoidal curve, with the exponential killing starting after around 3 minutes of incubation

Auxotrophic mutants of meningococci were isolated in a frequency approximately one fifth of that obtained with *E coli* Twenty such mutants were analysed with regard to growth factor requirements Some possible causes of the low mutation frequency are discussed

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STUDIES ON THE STREPTOMYCIN RESISTANCE SYSTEM OF *NEISSERIA MENINGITIDIS*

By

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Received 16 x 64

The development of bacterial resistance to streptomycin differs in many significant respects from that to other antibiotics There is a great variability in the phenotypic level of resistance obtained by one mutation only and high level resistance can easily be obtained in one mutational event (7, 23) Resistance to penicillin, erythromycin, and chloramphenicol, for instance, will occur in several steps with the initial mutations conferring resistance only to low concentrations of the antibiotic (7 6 2 25) Furthermore there are two fundamentally different types of insensitivity to streptomycin Streptomycin resistance (str r) and streptomycin dependence (str d) The first type will grow equally well with and without streptomycin in the medium, the second needs streptomycin for growth

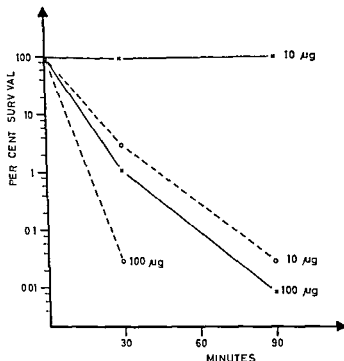
Genetic analyses of this resistance system have shown that sensitivity single-step high level resistance, and dependence are determined by multiple alleles of a single genetic locus (24, 11 27) Fine structure analysis in Pneumococcus by a transformational technique (26) has shown that all the studied sites of spontaneous mutations to str r are linked belonging probably to the same functional unit (cistron?) Loci on other places of the chromosome however have been described which will modify the expression of mutant loci that confer streptomycin resistance Such loci are for instance the modifier genes of *Pneumococcus* (4 26) and the suppressor genes of *E coli* (11)

In previous studies on the gentics of *N meningitidis* performed in our laboratory streptomycin insensitivity has regularly been *included* as a genetic marker (13 14 18 19) The present study has been directed towards the streptomycin resistance system *per se*, with the hope to get a better understanding of the genetic basis of resistance to streptomycin exhibited by this organism

MATERIALS AND METHODS

Bacterial s ... ined from Dr *Jyssum*
(12) Both h ... (14) In comparative
experiments ... (10)

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treptomyc n inactivation curves of meningococci grown in complete (o o) and minimal (— x — x —) med um when exposed to 10 and 100 µg f strej tomycin per ml

ml It is evident that the rate of killing is greatly reduced when the cells are grown in minimal medium and that in this environment they are almost resistant to the bactericidal action of as much as 10 µg per ml

Isolation of streptomycin insensitive mutants was always performed [illegible] of strep- [illegible] strepto [illegible] gar was [illegible] volume and incubation continued for another 60 hours This technique ensured that all mutations that had taken place during growth on the streptomycin free medium would remain in situ upon drug exposure and hence that all the colonies formed would represent independent [illegible]

[illegible] grew faster and formed greater colonies with an irregular border On the criteria of morphology 50 colonies were picked as resistant types and tested for growth on a streptomycin

Media Heart infusion broth (HIB) and agar (both from Difco) were used as liquid and solid complete media In some experiments blood agar plates were used as the solid complete medium The basal medium of meningococci has been de scribed by *Jyssum* (12) Streptomycin was used in the form of crystalline strepto mycin sulfate (Glaxo)

DNA preparations The method outlined follows in general that of *Marmur* (20) with the modifications of *Bovre* (5) Blood agar plates were heavily inoculated with the donor strain and incubated overnight The cells were harvested using around 3 ml of Na citrate buffer per plate Lysis of the cells was accomplished by adding 0 1 g of Na dodecylsulfate per 10 ml of bacterial suspension and placing the cells for 10 minutes at 56° C and subsequent 30 minutes at room temperature Na per chlorate was then added to a final concentration of 1 M and the whole mixture shaken with an equal volume of chloroform + isoamylalcohol (24 1 v/v) for 30 minutes at 5° C Subsequent centrifugation of the mixture at 5000 RPM for 30 minutes would leave the nucleic acids in the upper aqueous layer while the protein became concentrated at the interphase (first deproteinization) This layer was then pipetted off into another tube and mixed with the same volume of chloroform/iso amylalcohol The shaking was repeated for 15 minutes and the mixture centrifugated (second deproteinization) The layer containing the nucleic acids was removed and mixed with the double volume of ethyl alcohol (96 per cent) The nucleic acids would make a threadlike precipitate and float up to the surface where it was col lected with a sterile glass rod and drained free of excess alcohol After washing in 75 per cent alcohol the precipitate was finally dissolved in Na citrate buffer and stored at 5° C When DNA was to be isolated from a great number of different bacterial strains only one deproteinization was performed This turned out to be sufficient for genetic purposes No detectable decrease in biological activity was observed over a period of 6 months

Deoxyribonuclease (DNase) Sterile non crystalline deoxyribonuclease (Light) was dissolved 10 mg per ml in sterile 2 per cent gelatine solution and stored at 5° C (stock solution) Before use 1 part stock solution was mixed with 4 parts of 2 M $MgCl_2$ and 5 parts 2 per cent gelatine In most experiments a concentration of 10 µg of DNase per ml was sufficient to destroy all transforming activity of DNA present Concentrations beyond 100 µg per ml inactivated the cells rapidly

Genetic methods Two different techniques of transformation to streptomycin insensitivity were employed

a *Short time exposure* Cells were grown to log phase in HIB diluted 1 5 in pre warmed HIB containing 0 005 M $CaCl_2$ and DNA added to the desired concentration After 30–45 minutes at 37° C DNase was added and incubation continued for another 5 minutes to ensure the destruction of all DNA not irreversibly bound to recipient cells In control experiments the recipients were exposed to DNA pretreated with DNase for 5 minutes The cells were spread on complete agar plates and incubated 4–5 hours to ensure expression of the transformed marker Exposure to the drug was then accomplished by moving the agar to another agar plate of the same volume and composition containing twice the desired screening concentration of the drug Counting of transformant colonies was usually done after 30–40 hours When it was of interest to determine the resistance levels of the transformants the screening concentration of streptomycin was always 100 µg per ml After full growth of the transformed colonies they were either streaked or replicated on higher concentra tions of the drug

b *Long time exposure* The cells were inoculated together with the transforming DNA on complete agar plates and allowed to multiply for several hours When growth became clearly visible (4 6 hours) the agar was moved to the streptomycin containing medium

Other methods will be found in the result section

RESULTS

N meningitidis is highly sensitive to streptomycin and is rapidly in activated when exposed to the drug In Fig 1 are presented the inac tivation curves of actively dividing cells of strain 12 in complete and minimal medium when exposed to 10 and 100 µg of streptomycin per

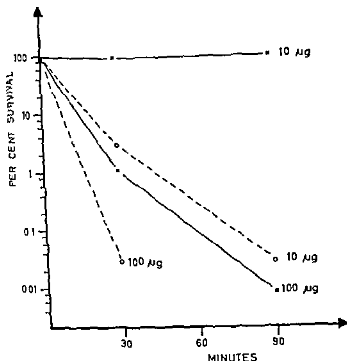


Fig 1

Streptomycin inactivation curves of meningococci grown in complete (- o - - o) and minimal (— x — x —) medium when exposed to 10 and 100 µg of streptomycin per ml

ml It is evident that the rate of killing is greatly reduced when the cells are grown in minimal medium, and that in this environment they are almost resistant to the bactericidal action of as much as 10 µg per ml

Isolation of streptomycin insensitive mutants was always performed on complete medium with a screening concentration of 100 µg of streptomycin per ml Sensitive cells were allowed to multiply on a streptomycin free medium until growth became clearly visible The agar was then moved to another agar plate of the same composition and volume and incubation continued for another 60 hours This technique ensured that all mutations that had taken place during growth on the streptomycin-free medium would remain "in situ" upon drug exposure, and hence that all the colonies formed would represent independent mutational events Both the resistant (str-r) and the dependent (str-d) variants were easily isolated with this technique At the end of the incubation period, these two types could be distinguished by their colony morphology The resistant type grew faster and formed greater colonies with an irregular border On the criteria of morphology, 50 colonies were picked as resistant types and tested for growth on a streptomycin-

free medium Only five of these turned out to be of the dependent variant *Miller & Bohnhoff* (21) observed the same morphological difference between their type A (resistant) and type B (dependent) variants

In order to determine the proportion of resistant to dependent types all mutant colonies per plate had to be examined in order to avoid the selective picking of the more prominent resistant types This was accomplished using the replica plating technique of *Lederberg & Lederberg* (17) The plates on which the mutant colonies grew, were replicated on HIB-plates and on HIB plates supplemented with 100 μg of streptomycin per ml Table 1 shows the results of experiments using both the highly and the non-transformable types of strain 12 It is clear that the dependent variant arises much more frequently, making up more than 90 per cent of the total number of streptomycin insensitive mutant colonies isolated on 100 μg per ml

In a comparative experiment, 136 mutant colonies of *E coli* were examined and their growth requirements of streptomycin determined Only 35 of these (ca 26 per cent) were of the dependent type

TABLE 1

Spontaneous Mutations to Streptomycin Insensitivity in N meningitidis

Strain*	Exp no	No of mutants tested	No of		Per cent str-d
			Str r	Str-d	
12, cp⁺	1	662	45	617	93 2
12, cp⁺	2	148	12	136	91 9
12 cp⁺	3	568	35	533	93 8
12, cp⁺	4	856	76	780	91 2
12 cp⁺	5	2198	122	2076	94 4
12, cp	1	184	19	165	89 7
12 cp	2	2337	140	2197	94 0
		6953	449	6504	93 54

All the isolations have been performed on complete media with a screening concentration of 100 μg of streptomycin per ml

* cp⁺ and cp designates the highly (cp) and non (cp) competent strain (14)

Properties of the str-r Mutants

The phenotypic level of resistance was roughly determined by streaking the mutants on a series of complete agar plates containing increasing concentrations of streptomycin This technique does not allow an ... of the resistance levels, but was considered suf- ... ifferences between independently isolated ... tested this way, only two types of resistance levels were found The first type (83 mutants) could grow on streptomycin concentrations up to 700 μg per ml, while the other (197 mutants) grew well on concentrations beyond 10,000 μg per ml

The competence of the resistant mutants was tested by streaking log-phase cells on minimal agar supplemented with 1 per cent broth



Fig 2

Screening method for competence using biochemical transformation Histidine requiring streptomycin resistant mutants have been streaked on basal agar plates containing 1 per cent broth, and DNA from a prototrophic strain has been added Only 2 of the 6 strains tested have been transformed to prototrophy by this method

(HIB) This concentration of broth will supplement a subliminal amount of histidine that will allow the cells to pass through a few divisions DNA extracted from a prototrophic strain was added and incubation continued for another 72 hours In Fig 2 an illustration of this screening method of competence is presented Prototrophic DNA has been added to all 6 strains inoculated on the plate, but only 2 have become transformed to prototrophy It became evident that mutations to both types of streptomycin resistance were frequently followed by a loss of competence Among 114 mutants of the high-level resistant type studied, 64 had lost competence, while 34 out of 68 low-level resistant types tested displayed a similar loss This means that more than 50 per cent of spontaneously arisen str r mutants of meningococci have lost competence

For genetic investigations, one competent and one incompetent mutant of each resistance level were isolated DNA was extracted from all 4 strains and sensitive cells transformed to resistance in "short-time-exposure" It was found that all the transformants tested (around 1000 in each experiment) had resistance levels identical to that of the donor strains, confirming that both types of resistance levels detected are due to single-site mutations

free medium Only five of these turned out to be of the dependent variant *Miller & Bohnhoff* (21) observed the same morphological difference between their type A (resistant) and type B (dependent) variants

In order to determine the proportion of resistant to dependent types all mutant colonies per plate had to be examined in order to avoid the selective picking of the more prominent resistant types This was accomplished using the replica plating technique of *Lederberg & Lederberg* (17). The plates on which the mutant colonies grew, were replicated on HIB-plates and on HIB plates supplemented with 100 µg of streptomycin per ml Table 1 shows the results of experiments using both the highly and the non-transformable types of strain 12 It is clear that the dependent variant arises much more frequently, making up more than 90 per cent of the total number of streptomycin insensitive mutant colonies isolated on 100 µg per ml

In a comparative experiment, 136 mutant colonies of *E coli* were examined and their growth requirements of streptomycin determined Only 35 of these (ca 26 per cent) were of the dependent type

TABLE 1

Spontaneous Mutations to Streptomycin Insensitivity in N meningitidis

Strain*	Exp no	No of mutants tested	No of		Per cent str-d
			Str r	Str-d	
12, cp^+	1	662	45	617	93 2
12, cp^+	2	148	12	136	91 9
12, cp^+	3	568	35	533	93 8
12, cp^+	4	856	76	780	91 2
12, cp^+	5	2198	122	2076	94 4
12, cp^-	1	184	19	165	89 7
12, cp^-	2	2337	140	2197	94 0
		6953	449	6504	93 54

All the isolations have been performed on complete media with a screening concentration of 100 µg of streptomycin per ml

* cp^+ and cp^- designates the highly (cp^+) and non (cp^-) competent strain (14)

Properties of the str-r Mutants

The phenotypic level of resistance was roughly determined by streaking the mutants on a series of complete agar plates containing increasing concentrations of streptomycin This technique does not allow an accurate determination of the resistance levels, but was considered sufficient for detecting greater differences between independently isolated mutants Among 260 mutants tested this way, only two types of resistance levels were found The first type (83 mutants) could grow on streptomycin concentrations up to 700 µg per ml, while the other (197 mutants) grew well on concentrations beyond 10,000 µg per ml

The competence of the resistant mutants was tested by streaking log-phase cells on minimal agar supplemented with 1 per cent broth

exist on the same genome but that they can replace each other This is the criterion of allelic markers as given by *Rotheim & Ravin* (26)

Properties of the str d Mutants

In many of the experiments to be described it was of importance to know the number of residual divisions undergone by dependent bacteria when transferred to a streptomycin free medium *Bertani* (3) has made extensive investigations with dependent mutants of *E coli* and found that the number of residual divisions depended both on the size of the inoculum and on the concentration of streptomycin of the medium in which the dependent cells grew before transfer Only the latter factor was examined in the present studies A dependent mutant was grown in HIB in a series of tubes containing respectively 5 25 100 500 and 1000 µg of streptomycin per ml After growth for 20 hours the cells were washed once in HIB and diluted 10^{-5} to ensure removal of all streptomycin not irreversibly bound to the cells The degree of division was determined by following the increase in viable cell count In Fig 3 the results of a typical experiment have been presented As expected the extent of residual growth is strongly influenced by the streptomycin concentration of the medium in which the cells grew before transfer The number of residual divisions is seen to be approximately 4 after growth in 1000 µg of streptomycin per ml while no significant growth is detected after transfer from a medium containing 5 µg of streptomycin per ml Another phenomenon apparent from Fig 3 is the rapid loss of viability of dependent cells when the residual growth has ended This is also observed in *E coli* (3) although in this species the time elapsing between the end of residual growth and loss of viability is considerably longer

In order to detect different types of dependent mutants of meningococci the following properties were studied

1 The phenotypic level of resistance
2 The minimum requirement of streptomycin
3 Growth on minimal media
4 Reversion rates to streptomycin independence
5 Competence

No differences in the first three propert [illegible]
different dependent mutants [illegible]
of streptomycin up to 2000 [illegible]
small background growth could be observed with the development of numerous mutant colonies resisting more than 10 000 µg per ml The [illegible]

The reversion from dependence to non dependence of streptomycin

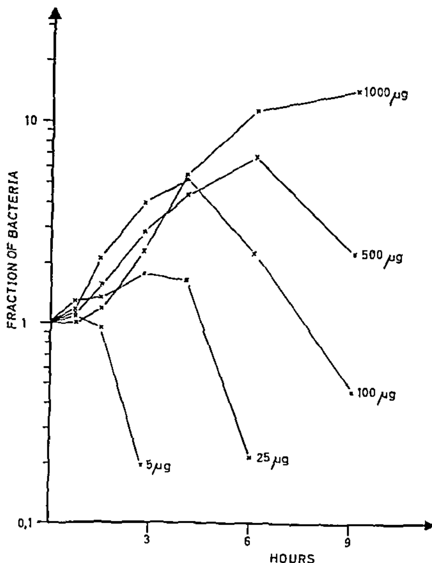


Fig 3

Growth of streptomycin dependent cells of meningococci in broth containing streptomycin The numbers indicate the concentration of streptomycin per ml the media in which the cells were grown before being deprived of the drug

If the two resistance levels described were due to non allelic mut loci, they should be able to coexist on the same genome This possibil was tested by transforming the competent low-level resistant type high-level resistance by DNA extracted from the high-level resista mutant DNA extracted from such transformants should in the case coexistence transform sensitive cells to two different levels of resi ance, depending upon which locus became integrated in the recipie genome One transformant from each of six independent experimen was selected and DNA extracted In every case it was found that the DNA preparations transformed only the high-level resistance to sen sitive recipients This will indicate that the two loci studied do not co

on which 0.1 ml of str-r DNA had been spread. During residual growth, competent mutants could be transformed from dependence to resistance and form numerous independent colonies. Fig. 4 illustrates this type of transformation. Residual growth on the plate 3 hours after replication is still sufficient both for transformation and for phenotypic expression of the transformed marker. This is in good agreement with the results in Fig. 3, where it is seen that when a dependent mutant is grown in a medium containing 100 μg of streptomycin per ml, the residual growth will continue for approximately 4 hours after removal of the drug. Among the 135 mutants tested for competence, 23 had lost the ability of being transformed as measured both by the transformation to prototrophy and to streptomycin resistance. This loss is lower than the one found among the resistant mutants, but is significantly higher than the spontaneous loss found in the sensitive, parental strain (14).

Excluding competence, the properties of the dependent mutants examined so far have failed to reveal any differences in the phenotype of the mutants studied. So far, therefore, there is nothing to indicate that different mutations are involved in the production of this phenotype. The crucial test from the genetic point of view, however, would be to examine the possibility of obtaining independent recombinants when utilizing independently isolated mutants as donors and recipients in transformation studies.

With this aim in mind DNA was prepared from 10 mutant strains (4 of which were incompetent) and 30 different competent strains selected to serve as recipients. The "long time exposure" transformation technique was utilized. Young recipient cultures grown on 100 μg streptomycin per ml were streaked or replicated on streptomycin free agar plates and DNA from the donor strains added immediately. The results were altogether negative. In none of the 300 different combinations tested could any transformants be detected.

Hashimoto (11) found that when dependent strains of *E. coli* were transduced to independence by phage from resistant strains, a small fraction of the transductants were of the sensitive phenotype, representing recombination between the two markers. To see if a similar phenomenon occurred in meningococci, competent dependent mutants were transformed in "short time exposure" by resistant DNA using streptomycin free agar plates as the selective medium. The transformation frequencies obtained under these circumstances was low, being around one transformant per 10^6 cells plated. No sensitive phenotype [illegible]

[illegible] technique, therefore,

[illegible] process between the

The transformation of dependent cells by resistant DNA described above takes place during the residual growth of the recipients. It was of interest to see if a similar transformation could be achieved with



Fig 4

Transformation of streptomycin dependent mutants of meningococci to independence by str–r DNA A confluent growth of dependent cells on a medium containing 100 µg of streptomycin per ml has been replicated on a streptomycin-free medium and 0 1 ml of DNA added after the number of hours indicated on the figure Residual growth is seen to be sufficient both for transformation and phenotypic expression up to 3 hours after replication

was examined using 30 different mutant strains I some experiments the bacteria were grown on agar plates containing varying amounts of streptomycin, suspended in saline and plated on streptomycin-free medium If the growth took place in liquid medium, the cells were first spun down and washed once in saline to ensure removal of extracellular streptomycin In all the strains tested it turned out to be very difficult to obtain independent mutants Plating of more than 10^{10} cells only rarely resulted in one or two independent colonies Pregrowth of the cells was performed in 5, 25, 50, and 100 µg of streptomycin per ml and the cells tested for reversion did undergo significant residual growth, which at heavy inocula could directly be observed as a thin layer of bacterial growth on the streptomycin free agar plate From several experiments, 20 revertants were isolated and found to be of the sensitive phenotype only

Competence of the dependent mutants could be examined by two different techniques The first was that illustrated in Fig 2, using minimal agar supplemented with 100 µg of streptomycin per ml and 1 per cent HIB In the other the mutants were streaked on HIB-agar plates containing 100 µg of streptomycin per ml After around 10 hours of incubation, the plates were replicated on a streptomycin-free plate

on which 0 1 ml of str r DNA had been spread During residual growth, competent mutants could be transformed from dependence to resistance and form numerous independent colonies Fig 4 illustrates this type of transformation Residual growth on the plate 3 hours after replication is still sufficient both for transformation and for phenotypic expression of the transformed marker This is in good agreement with the results in Fig 3 where it is seen that when a dependent mutant is grown in a medium containing 100 μg of streptomycin per ml, the residual growth will continue for approximately 4 hours after removal of the drug Among the 135 mutants tested for competence, 23 had lost the ability of being transformed as measured both by the transformation to prototrophy and to streptomycin resistance This loss is lower than the one found among the resistant mutants, but is significantly higher than the spontaneous loss found in the sensitive, paren tal strain (14)

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The transformation of dependent cells by resistant DNA described above takes place during the residual growth of the recipients It was of interest to see if a similar transformation could be achieved with

DNA isolated from a sensitive strain This has been accomplished in *E coli* by a transductional technique (11) Experiments were performed with 30 different dependent mutants as recipients and with pregrowth on 50, 100 and 500 μg of streptomycin per ml Transformation of dependent cells back to independence by DNA from sensitive cells could not be detected in any instance, even though the recipients were easily transformed by resistant DNA

DISCUSSION

Fig 1 shows the decreased sensitivity of meningococci to the action of streptomycin when grown in minimal media *Engelberg & Artman* (9) have shown that the streptomycin uptake of dependent cells of *E coli* is dependent upon the ionic strength of the medium with a maximum uptake in salt free solutions Indications that this is true also for resistant and sensitive strains of *E coli* are given by *Anand et al* (1) Thus the decreased sensitivity of meningococci to the action of streptomycin in minimal medium might simply reflect a lowered uptake of the drug in this environment

The proportion of dependent variants among the streptomycin insensitive mutants of *N meningitidis* isolated on 100 μg per ml is much higher than the one obtained using the strain K 12 of *E coli* The proportion of dependent mutants in *E coli B* has been reported to be around 60 per cent (8, 22) This higher proportion in meningococci might either be due to a higher mutability of the dependent locus or to some selective disadvantage of the str-r mutants One possibility pointing to the latter hypothesis is the process of "*in vivo* transformation" which is very pronounced in meningococci (13, 18) During growth on the streptomycin free medium the resistant variants (in contrast to the dependent) will continue to grow after phenotypic expression and may be transformed back to sensitivity by the surrounding cells If both the sensitive and the resistant alleles were present in the same cell at the time of drug-exposure, this particular mutant gene would be eliminated due to the dominance of the sensitive allele (16) Dependent mutants, however, will stop to grow immediately after expression and the elimination process should be considered to be much less effective in this instance However, the fact that insensitive mutants isolated from an incompetent strain show the same high frequency of dependent variants points against this theory as these cells are known not to be subject to "*in vivo* transformation

The different streptomycin insensitive mutants of meningococci studied in the present paper exhibit a much greater homogeneity than that observed among mutants from other species The two different types (str-r and str-d) will in the following be discussed separately

Only two phenotypically distinct levels of resistance were found among 260 mutants tested One was at 700 μg of streptomycin per ml

(low level), while the other was beyond 10 000 µg per ml (high level) This is in contrast to conditions in pneumococci (4) and *E coli* (7, 23), where single-step resistant mutants show a much greater variation in the maximum concentration of streptomycin they are able to resist It must be emphasized that the method used for screening the pheno typic level of resistance does not allow any smaller differences to be detected However due to physiological variations in the resistance levels exhibited by one and the same mutant (26), genetically distinct mutants with close phenotypic levels of resistance are very difficult to distinguish

The two types of resistance levels obtained have been shown to be due to allelic mutational sites Transformation of the low resistant type to the high resistance level gives transformants that possess only the latter marker The two markers do not coexist in the same genome, but can replace each other, which is the criterion of allelic markers as given by *Rotheim & Ravin* (26) The same authors have shown that in pneumococci, the level of resistance conferred by the mutant site is probably correlated to the size of that particular site In meningococci such considerations must await the isolation of mutants with genotypes differing from the two described in the present paper

Apart from competence, no differences have been observed among the dependent mutants of meningococci studied This again is in contrast to the situation in *E coli* Some of the differences found among dependent mutants of the latter organism are the lower concentration of streptomycin allowing growth of the dependent cells (24), the con centration of streptomycin the cells are able to resist (11), the require ment of additional growth factors (24) and the reversion rates to non-dependence (3 15)

With regard to the upper and lower concentrations of streptomycin allowing growth of the mutants and the ability to grow on minimal medium there is no doubt about the similarities among the mutants studied When it comes to the study of reversion rates to independence an [illegible]
tar [illegible] 3
m [illegible]
Even after growth at 500 µg of streptomycin per ml the cells are *not* saturated with the drug as indicated by the longer residual growth when pregrown at 1000 µg per ml This intracellular pool might ac count for the failure of transforming dependent cells to independence by DNA from a sensitive strain, although the recipients were highly competent as measured by the ability to be transformed by DNA from str r mutants If sensitivity is assumed to be dominant to dependence, the entry of this gene into a dependent cell will express itself imme diately and the cell will be killed by the pool of streptomycin present in the cell It is known from experiments with *E coli* (27) that when dependent cells are deprived of the drug there is an increasing abnorm-

ality in the macromolecular composition of the cell It might well be, therefore, that when the intracellular pool of streptomycin has decreased to a level permitting the survival of a change to the sensitive phenotype, the period of competence is ended due to the gross alterations in the composition and metabolic activities of the recipient

If the reversions of dependent cells are all to the sensitive phenotype and a possible recombination between different dependent mutants also restore this phenotype, then the intracellular pool of streptomycin might account for both the low reversion frequencies obtained and for *the lack of obtaining independent recombinants* The few revertants obtained were all of the sensitive phenotype and might represent mutations that had taken place immediately before exhaustion of the streptomycin pool In the strain Sd 4 of *E coli B*, approximately 30 per cent of the mutations to independence restored the resistant phenotype (3) According to the present theory this type of reversion can not exist in the meningococcal strains tested Nothing can be said, therefore, about the identity of the different dependent meningococcal mutants on the basis of reversion frequencies and the studies on recombination between them

The failure to obtain sensitive recombinants between the dependent and resistant mutants would indicate that these markers were allelic However, again the pool of streptomycin inside the cell might be responsible for the negative results Because dependent cells always had to be used as recipients in such studies, any recombinant would remain undetected due to the pool of streptomycin present at the time of transformation

SUMMARY

Studies on mutations to streptomycin insensitivity in *N meningitidis* have been performed With a screening concentration of 100 μg of streptomycin per ml, 6 5 per cent of the spontaneously arisen mutants were of the resistant type (str-r) while 93 5 per cent belonged to the dependent variant (str-d)

Among 260 str-r mutants studied, only two distinct levels of resistance were found By transformation studies, the two loci responsible were shown to be allelic More than 50 per cent of the mutants studied had lost competence in transformation tests

No differences could be detected among 135 str-d mutants studied with regard to the maximal and minimal concentrations of streptomycin allowing growth No mutant strain showed additional growth factor requirements on minimal medium The reversion rates to independence were very low, and no recombination could be detected among distinct mutants to produce the independent phenotype The mutants could be transformed to independence by DNA carrying the resistant, but not the sensitive, streptomycin allele No sensitive phenotypes could

be detected among transformants produced by str-r DNA Some possible explanations of these phenomena are discussed

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BRIEF REPORT

PERMEABILITY INCREASING EFFECT OF CELL FREE ASCITIC FLUID FROM BERGEN A4 ASCITES CARCINOMA

By *Stein Thunold*

During growth of ascitic tumours in mice large amounts of fluid accumulate in the peritoneal cavity. This increase in fluid has in the case of the non-specific Ehrlich's ascites carcinoma been shown to be directly proportional to the increase in total number of tumour cells (3, 7). *Straube* (6) found that the ascitic fluid originated at least in part from the blood. The finding of a relative excess of the albumin fraction in the cell free fluid (4, 7) giving an A/G ratio different to that of normal serum indicates an active process. The finding of large amounts of red blood cells is also important (1). These facts suggest an increased capillary permeability in the peritoneum. The following experiment was designed to see if this is so.

Material and methods.—Animals. Adult male and female mice from a non-inbred strain were used. They were fed on commercial pellets and water, which were withdrawn during the experiment.

Tumour. Cell free fluid from the Bergen A4 (BA4) mouse ascites carcinoma was used (2). This tumour can be serially transplanted in different strains of mice and must thus be regarded as a non-specific tumour.

Experimental procedure. As accumulation of dye labelled plasma proteins in the peritoneal cavity can be used for studying the permeability of the peritoneal vessels, the procedure described by *Northover* (5) was used with some modifications. Northover dilutes his test substances in 4 ml saline and injects this intraperitoneally. Within the next 30 seconds he injects Evans blue via a lateral tail vein. After 1 hour the peritoneal fluid is removed, centrifuged and the optical extinction of the supernatant is measured. In the present experiment cell free ascitic fluid from a 12 day transplant of the tumour was tested. Pooled serum from the same strain and saline were used as controls. The test fluids were injected intraperitoneally in doses of 0.5 ml followed half an hour later by 4 ml of saline. This was done to allow the test substance to act undiluted. Otherwise Northover's procedure was followed.

Results. The results are shown in Fig. 1 which gives the calculated values for the total amount of protein bound dye accumulated in the peritoneal cavity. This is a measure of the capillary permeability. Analysis of variance followed by a test of all comparisons among means was carried out on square root transformed values. The amount of intraperitoneal dye following injection of ascitic fluid was significantly greater than that following either serum or saline ($P < 0.005$). The difference between the serum and saline groups was not significant. Thus the experiment shows that the ascitic fluid contained a substance(s) that increased capillary permeability.

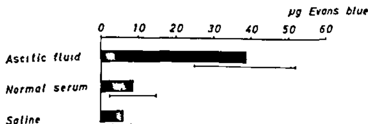


Fig. 1.

Total amount of Evans blue accumulated in the peritoneal cavity in µg. Mean ± SD (|—|). Three male and 3 female mice in each group.

Received 10.ii.65 from The Gade Institute, Department of Pathology, The University, Bergen, Norway (Head: Professor *E. Waaler*, M.D.)

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BRIEF REPORT

ISOLATION OF THE TRIC AGENT FROM EYE AND UROGENITAL TRACT OF TRACHOMA PATIENTS IN DENMARK

By *C H Mordhorst*

In a previous article (1) the isolation of TRIC agents from the eye of newborn babies with inclusion blennorrhoea has been reported In that study a TRIC agent was also recovered from the cervix of one of the mothers The presence of a TRIC agent in the urogenital system has recently been reported by *Jones et al* (2) who obtained an isolate from urethral scrapings of a father to a newborn baby suffering from inclusion blennorrhoea

In the present study is reported the isolation of TRIC agents from the eye and urethra of one patient with a trachoma like infection and from the vagina of another patient with typical trachoma Material for laboratory examinations was collected as epithelial scrapings from the eye and urogenital tract The methods

corneal lesions congestion of vessels at upper limbus and penetration of a few vessels 1 2 mm into the cornea and the case was classified as atypical trachoma

measured in the serum o was

Received 14 iii 65 from *The Influenza Department Statens Seruminstitut Copenhagen*

The strains isolated from the 2 patients were usually recognized in the 2–3rd yolk sac passage and the embryos were killed in this or the following passages The *impression smears of the yolk sac membrane disclosed numerous free elementary* bodies The egg LD_{50} of the isolates after establishing was of the order of $10^{3.5}$ to $10^{5.5}$ per ml All strains have so far been through at least 8–10 egg passages Antigen prepared from the isolates by the method described by *Volkert & Moller Christensen* (4) fixed complement with lymphogranuloma venereum antisera to full titre The conjunctival isolate as well as the isolates from the urogenital tract were inoculated onto the conjunctival sac of monkeys (*M cynomolgus*) and caused a follicular conjunctivitis with typical inclusion bodies The agents could be re isolated from the animals' eyes The clinical course in the monkey was essentially the same whether the agent derived from the patient s eye or from the urogenital tract (5)

The diagnosis of trachoma in two Danish patients has thus been established on clinical and microscopical findings and confirmed by the isolation of a TRIC agent from the eye of one of the patients and by a TRIC agent isolation from the urogenital tract of both patients It should be mentioned that both patients previously had been treated locally in the eye with various antibiotics with temporary success

taken place

al tract in both

ions were caused

by transfer of infectious material from the urogenital tract to the eye

Summary Two active cases of trachoma in Denmark are reported An agent belonging to the TRIC (trachoma inclusion conjunctivitis) group was isolated from the eye and urethra *of one patient and from the vagina of the other The possibility* that the urogenital tract is a reservoir for recurrent eye infections in the two patients is mentioned

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Acta path et microbiol scandinav 63 639–640, 1965

BRIEF REPORT

ROLE OF FRESH HUMAN OR GUINEA-PIG SERUM IN THE INDIRECT FLUORESCENT ANTIBODY TEST FOR *MYCOPLASMA PNEUMONIAE* ANTIBODIES IN HUMAN SERA

By K Lind

In 1959 *Liu et al* (5) reported that the addition of fresh normal human or guinea-pig serum to sera before testing for antibodies against Eaton agent by *Coons'* indirect fluorescence technique, clearly enhanced the reaction *Liu* showed that this

reaction Experiments were therefore performed to elucidate the role of fresh guinea

and results

Sera with titres from 640 to 2560 from patients with atypical pneumonia, sera with titres of about 40 from healthy donors, and negative control sera with a titre of < 10 were heat inactivated at 56° C for 30 min before use

Pooled fresh *guinea pig serum* was used untreated or heat inactivated

Fresh *human sera* from healthy donors with an anti M p titre of < 20 before inactivation were used immediately after clotting and centrifugation at 22° C, either untreated or inactivated

As a *diluent* for the titration of human sera in the first experiments an isotonic ... d This was later exchanged with an ... (VB++) to give optimal conditions ... mplement fixation another veronal

The following conjugates were ...
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Titration and score Sera were titrated by two or four fold serial dilutions in buffer and the reactions were read as 4 3, 2, 1 or 0 (4 = maximal 0 = negative fluorescence) The score of a particular serum is the sum of the readings of all its dilutions

Received 10 III 65 *from the Department of Toxoplasmosis & Viral Diseases* (Head J Chr Siim M D), *Statens Seruminstitut, Copenhagen*

TABLE 1

Role of Guinea Pig Complement in the Indirect Fluorescent Antibody Test for Human Mycoplasma Pneumoniae *Antibodies*

Human sera diluted in	Scores	
	Horse anti human globulin cojugate	Horse anti guinea pig serum conjugate
Veronal buffer with fresh guinea pig serum	13	13
Veronal buffer with inactivated guinea pig serum	17	0
Veronal buffer without guinea pig serum	18	0

An explanation of the lower scores when using fresh GPS in the diluent for titration of sera might be that guinea pig complement is fixed to the *M p* antibodies which have been attached to the antigens leading to a partial blocking of the subsequent reaction of conjugated anti human globulin with antibody In order to examine if such a fixation of guinea pig complement had taken place two series of three

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sera diluted in fresh GPS tested with conjugated anti human globulin is in contrast to a high score for the same serum dilutions tested with anti guinea pig conjugate The score with this conjugate was 0 for serum diluted in buffer with inactivated GPS or without GPS The possibility that supposed *M p* antibodies in the GPS might have attached to the antigen by fixation of complement in the fresh GPS was ruled out in other experiments where the *M p* antigen was incubated with serial dilutions of both fresh and inactivated GPS in VB After treatment with conjugated anti guinea pig serum (AGPH PLB) no score was recorded in either of the serial dilutions

Comments Evidence is provided which suggests that the observed inhibition by fresh guinea pig serum as a diluent for human sera tested with conjugated horse anti human globulin is due to a partial blocking of the *M p* antibodies by guinea pig complement

The results of *Liu* (6) showing an enhancement by fresh normal guina pig or human serum were obtained with a fluorescent anti human globulin from rabbit when human antibodies were tested

Further experiments were therefore performed employing fresh guinea pig and human sera as diluents for titrations of human antisera and staining with different anti human globulin conjugates from various animals The inhibition by fresh guinea pig serum was a constant observation although varying in degree with the

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INDEX

VOL 63 FASC 1–4 1965

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Veronal buffer with inactivated guinea pig serum	17	0
Veronal buffer without guinea pig serum	18	0

An explanation of the lower scores when using fresh GPS in the diluent for titration of sera might be that guinea pig complement is fixed to the *M p* antibodies which have been attached to the antigens leading to a partial blocking of the subsequent reaction of conjugated anti human globulin with antibody. In order to examine if such a fixation of guinea pig complement had taken place two series of three different titrations were carried out as described above namely in VB + a) with fresh GPS b) with inactivated GPS and c) without GPS. Conjugated anti human globulin (*AHGH PLB*) was added to one of the series, conjugated anti guinea pig serum (AGPH PLB) was added to the other. As seen in Table 1 a reduction in score for sera diluted in fresh GPS tested with conjugated anti human globulin is in contrast to a high score for the same serum dilutions tested with anti guinea pig conjugate. The score with this conjugate was 0 for serum diluted in buffer with inactivated *GPS* or without *GPS*. The possibility that supposed *M p* antibodies in the GPS might have attached to the antigen by fixation of complement in the fresh GPS was ruled out in other experiments where the *M p* antigen was incubated with serial dilutions of both fresh and inactivated GPS in VB+. After treatment with conjugated anti guinea pig serum (AGPH PLB) no score was recorded in either of the serial dilutions.

Comments. Evidence is provided which suggests that the observed inhibition by fresh guinea pig serum as a diluent for human sera tested with conjugated horse anti human globulin is due to a partial blocking of the *M p* antibodies by guinea pig complement.

The results of *Liu* (6) showing an enhancement by fresh normal guinea pig or human serum were obtained with a fluorescent anti human globulin from rabbit when human antibodies were tested.

Further experiments were therefore performed employing fresh guinea pig and human sera as diluents for titrations of human antisera and staining with different anti human globulin conjugates from various animals. The inhibition by fresh guinea pig serum was a constant observation although varying in degree with the ... r in

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